NORTON FELIPE DOS SANTOS SILVA

Morfologia, química e função na glândula da membrana artrodial e química de hidrocarbonetos cuticulares em um opilião (arachnida, opiliones)

Tese apresentada ao Programa de Pós-Graduação em Biologia de Sistemas do Instituto de Ciências Biomédicas I da Universidade de São Paulo, para a obtenção do Título de Doutor em Ciências

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Citação

Funk da dissertação

Aluno tá a milhão
Preparando mais uma versão
A cabeça fica louca por que o texto
ainda não tá bom
A cabeça fica louca
Sabe que tá meia boca
Atividade 24 horas
Não sai mais linha

E discussão? Te deixa como?

Te deixa louco (Refrão)
Quase se matando
Essa discussão tá apenas começando
Te deixa louco, louco
Quase se matando
Essa discussão tá apenas começando

É vou te falar a verdade
Melhor é pedir mais prazo
Para a comunidade
A banca é foda
Isso me incomoda
Página um, página dois
A escrita toda zoada

Ontem eu vi
Os resultados da análise
Gráfico feio e torto
Tabela Errada

O *et al* se entortando
Outros nem tanto
Prá lá e pra cá perguntei
O que que era, resposta clara

Era o tranca tese, que tava na pista
Se tu vacilar
Teu nome vai entrar na lista

Refrão

Te deixa louco
Quase se matando
Essa discussão tá apenas começando
Te deixa louco, louco
Quase se matando
Essa discussão tá apenas começando

Autor: Norton F S Silva

(Paródia - Mc B.A)

SILVA, NFS. **Morfologia, química e função na glândula da membrana artrodial e química de hidrocarbonetos cuticulares em um opilião (arachnida, opiliones)**. 2023. 86 f. Tese (Doutorado em Biologia de Sistemas) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2023.

O processo de artropodização inclui a evolução de um exoesqueleto rígido que está na origem de uma série de modificações morfológicas. As pernas articuladas, por exemplo, são possíveis justamente por haver partes rígidas que permitem a sustentação do corpo e ao mesmo tempo a inserção de musculatura internamente. Enquanto músculos, pressão hidráulica e tendões comandam os movimentos, uma vasta gama de estruturas sensoriais externalizadas no exoesqueleto recebe estímulos do ambiente e glândulas diversas atuam no bom funcionamento, proteção, balanço hídrico e comunicação entre indivíduos. As articulações das pernas precisam permitir movimento entre as partes rígidas que ligam. Estas áreas do corpo são revestidas por uma membrana artrodial cujo revestimento mais delgado possibilita movimento. Estas membranas possuem aberturas glandulares conhecidas unicamente por fotos de microscopia eletrônica de varredura. No corpo principal deste doutorado, então, investimos no aprofundamento do conhecimento sobre esta região, buscando entender sua estrutura interna, química de seus componentes e compreensão da função de tais químicos. Do ponto de vista morfológico, descrevemos os componentes teciduais e celulares da membrana artrodial por meio de técnicas diversas como microscopia eletrônica de varredura, óptica e de transmissão. Para a descrição química, utilizamos testes histoquímicos, de cromatografia líquida de alta performance, eletroforese e espectrometria de massas. Já para o estudo da função das secreções, quantificamos os componentes por meio de um espectrofotômetro, fracionamos e analisamos os componentes com cromatografia líquida de alta performance, incubamos e determinamos o grau de inibição de crescimento de fungos e bactérias. Os resultados obtidos após o uso desta diversidade de técnicas são descritos em detalhes em cada capítulo e seus respectivos resumos. Mas obtivemos a primeira descrição de uma glândula de membrana artrodial, proteômica e demonstração de ação antifúngica e antibacteriana nestas secreções em aracnídeos da ordem Opiliones. Em uma parte adicional deste doutorado, investigamos uma propriedade do exoesqueleto também completamente desconhecida em opiliões (Opiliones): os hidrocarbonetos

cuticulares. Estes primariamente minimizam perda d´água, mas também são conhecidos por possuírem inúmeras funções nas interações de artrópodes com outros animais. Tipicamente, são químicos de contato que participam em reconhecimento específico e sexual, além de prover inúmeras informações sobre o indivíduo. Em opiliões, há amplas evidências da importância da quimiorrecepção de contato, tanto do ponto de vista do emissor quanto do receptor. Entretanto, não tínhamos ideia de quais químicos atuariam nestas interações. Descrevemos, então, os hidrocarbonetos cuticulares de ambos os sexos em uma espécie de opilião, tendo detectado inúmeros químicos utilizados em comunicação química em outros taxa, além de diferença entre machos e fêmeas. A tese está dividida em 4 capítulos independentes, mas sob o guarda-chuva de estrutura cuticular em Opiliones. Cada um consiste em uma publicação. O primeiro está publicado, o segundo está aceito, o terceiro e o quarto estão submetidos.

Palavras-chave: membrana intersegmentar, lubrificação, opiliões, feromônio de contato, comunicação química

SILVA, NFS. **Morphology, chemistry and function in the arthrodial membrane gland and cuticular hydrocarbon chemistry in a harvestman (arachnida, opiliones)**. 2023. 99 f. Ph. D. these (Doctorate in Systems Biology) - Instituto de Biomedical Sciences, University of São Paulo, São Paulo, 2023.

The process of arthropodization includes the evolution of a rigid exoskeleton that is at the origin of a series of morphological modifications. Articulated legs, for example, are possible precisely because there are rigid parts that allow the body to be supported and at the same time allows insertion of muscles internally. While muscles, hydraulic pressure, and tendons command the movements, a wide range of sensory structures externalized in the exoskeleton receive stimuli from the environment, and various glands play a role in the proper functioning, protection, water balance, and communication between individuals. The joints of the legs need to allow movement between the rigid parts they connect. These areas of the body bear an arthrodial membrane whose thinner lining enables movement. This membrane has glandular openings known only from scanning electron microscopy micrographs. In the main body of this PhD, then, we invested in deepening our knowledge of this region, seeking to understand its internal structure, chemistry of its components, and understanding the function of such chemicals. From a morphological point of view, we described the tissue and cellular components of the arthrodial membrane using various techniques such as scanning, optical and transmission electron microscopy. For the chemical description, we used histochemical tests, high performance liquid chromatography, electrophoresis and mass spectrometry. To study the function of the secretions, we quantified the components using a spectrophotometer, fractionated and analyzed the components using high performance liquid chromatography, incubated and determined the degree of inhibition of fungal and bacterial growth. The results obtained after using this diversity of techniques are described in detail in each chapter and their respective summaries. But we have obtained the first description of an arthrodial membrane gland, proteomics and demonstration of antifungal and antibacterial action on these secretions in arachnids of the order Opiliones. In a further part of this PhD, we investigate a property of the exoskeleton also completely unknown in harvesters (Opiliones): the external covering of the body by cuticular hydrocarbons. These primarily minimize water loss, but are also known to have numerous functions in arthropod interactions with other animals. Typically, they are

contact chemicals that participate in specific and sexual recognition, in addition to providing a wealth of information about the individual. In harvesters, there is ample evidence for the importance of contact chemoreception from both the sender and receiver perspectives. However, we had no idea which chemicals would be relevant in these interactions. We therefore described the cuticular hydrocarbons of both sexes in one species of harvester, having detected numerous chemicals used in chemical communication in other taxa, as well as a difference between males and females. The thesis is divided into 4 independent chapters, but under the umbrella of cuticular structure in Opiliones. Each consists of one publication. The first is published, the second is accepted, the third and fourth are submitted.

Keywords: intersegmental membrane, lubrication, harvestman, contact pheromone, chemical communication

SUMÁRIO

INTRODUÇÃO GERAL

A cutícula é uma camada que cobre o corpo dos artrópodes, e suas principais funções são dar a forma do animal, evitar perda d'água, abrigar estruturas sensoriais e glandulares, proteção contra predadores e agentes infecciosos (Chapman 1998, Vincent 2002). Além disso, a cutícula possui moléculas que podem servir para o reconhecimento de indíviduos de uma espeće, bem como reconhecimento do sexo do organismo durante o processo de comunicação química em que um organismo reconhece o outro como membro de sua espécie, o sexo do mesmo ou como um indivídio estranho (Wyatt 2014). A cutícula apresenta diferentes propriedades mecânicas dependendo da sua localização no corpo. Nesse sentido, a cutícula presente em regiões entre os segmentos do corpo, apresentam maior maciez, menor esclerotização e propriedades elásticas quando comparadas com as regiões fora dos segmentos (Gorb 2002, Moussian 2013). O exoesqueleto e muitos órgãos sensoriais dos artrópodes são formados por cutícula, e esta é subdividida em duas camadas principais chamadas epicutícula e procutícula. Além disso, existem outras duas camadas que compreendem a epiderme. A primeira camada contém células epidermais que produzem a cutícula e outras secreções. A segunda camada é chamada de membrana basal que é localizada abaixo das células epidermais (Moussian 2013). Epicutícula é a camada mais fina e externa da cutícula e cobre a superfície dos animais. Ela é dividida em subcamadas, chamadas 1) subcamada de cimento, 2) sub camada de cera, 3) epícutícula externa e 4) epícutícula interna.

As principais funções da epicutícula são homeostase da água, estabelecer a forma do exoesqueleto, transporte de fluídos da muda e secreções epidérmicas. Além disso, serve como reservatório químico para armazenamento de resíduos metabólicos, hormônios e secreções defensivas (Betz 2010). Procutícula é a parte da cutícula que contém quitina (polissacarídeo) e também é composta por produtos secretados da epiderme. Ela é dividida em dois principais componentes, a quitina e proteínas. A quitina é um polissacarídeo constituído por um polímero de cadeia longa de N-acetilglicosamina e tem forma cristalina no exoesqueleto dos artrópodes. Existem 3 formas formas de quitina (α, β e γ-quitina) e elas são classificadas como polímeros viscoelásticos, que nos artrópodes ocorrem como feixes de microfibrilas associados a proteínas (complexo quitina-proteína) (Gorb 2002, Vincent 2002).

Propriedades mecânicas da cutícula

Considerando propriedades mecânicas da cutícula, ela pode ser funcionalmente dividida em cutícula sólida, cutícula artrodial membranosa e/ou cutícula contendo resilina (Moussian 2013). A cutícula sólida é o tipo mais duro de cutícula, esta não apresenta flexibilidade e sua rigidez aumenta conforme a quantidade de proteína não extraível de hidróxido de sódio (NaOH) aumenta (Andersen e Barret 1971). Esse tipo de proteína apresenta maior grau de esclerotização, processo este que diminui a capacidade de deslizamento das proteínas da cutícula e consequentemente diminui a flexibilidade delas (Moussian 2013). Esse tipo de cutícula compõem os chamados escleritos duros dos artrópodes. A membrana artrodial é um tipo de cutícula altamente flexível, extensível e resistente e que conecta os escleritos duros. Essa cutícula é encontrada em junções móveis das pernas, antenas, órgãos copuladores, ovipositor, pescoço, partes da boca e segmentos do corpo (Gorb 2006, Michels e Gorb 2011, Michels et al 2016). Existem dois tipos de membrana em insetos. A primeira é chamada de membrana altamente extensível e esta pode ser estendida mais de 10 vezes o seu comprimento (Vincent e Woods 1972, Vincent 1981). A segunda é chamada de cutícula membranosa laminada dobrada que tem um grau de extensão menor que a primeira (Vincent 1981, Hackman e Goldberg 1987). Suas principais funções incluem, conectar elementos esclerotizados do exoesqueleto, permitir movimento desses elementos e estender quando um aumento de volume é necessário e também função defensiva através de protuberâncias em miniaturas. Estas membranas também possuem uma proteína elástica chamada de Resilina (Michels et al 2016). A resilina é uma proteína elastomérica presente em determinadas cutículas que apresenta características elásticas que funcionam como molas sob tensão e compressão (Frazier et al 1999, Michels et al 2016). Ela possui uma estrutura molecular em rede tridimensional, é composta por cadeias polipeptídicas enroladas randomicamente orientadas que são ligadas covalentemente entre si em intervalos regulares por aminoácidos fluorescentes (ditirosina e tritirosina) e glicina (Andersen 1963, 2010). Essa estrutura forma uma rede estável com alta capacidade de mobilidade e flexibilidade (Neff et al 2000). Cutículas contendo resilina são também chamadas de “cutícula tipo-borracha" em contraste a cutícula sólida e pode ser distendida reversivelmente 2 vezes do seu tamanho inicial e formam compostos com outras proteínas e/ou fibras de quitina

(Weis-Fogh 1960). Ela pode ser encontrada em associação com diversas estruturas dos artrópodes, incluindo pedipalpo, lentes de olhos compostos, tendões, mecanorreceptores, mecanismo de pulo e salto, mecanismo de alimentação de insetos sugadores de sangue, órgão produtor de som, cutícula abdominal e perna locomotivas (Rothschild et al 1975, Hepburn e Chandler 1976, Edwards, 1983, Neff et al 2000, Michaels et al 2016). As principais funções da resilina incluem, armazenamento de energia elástica em sistemas de saltos, redução de fadiga de asas dobráveis de insetos voadores, aumento de adaptabilidade de fixação de estruturas a superfícies irregulares, geração de flexibilidade de articulações em asas de insetos voadores e flexibilidade em juntas de pernas locomotoras (Michels et al 2016, Gorb 2002).

Glândulas cuticulares

As glândulas são estruturas responsáveis por gerar fluidos secretórios necessários para a função e funcionamento de tecidos alvos. Elas são responsáveis por produzir feromônios, substâncias adesivas, antibióticos, enzimas digestivas, substâncias defensivas e lubrificantes (Betz 2010, Billen e Sabotnik 2015, Wolff et al 2016). Baseado nesta diversidade de contextos biológicos e grupos sistemáticos, estruturas glandulares são encontradas em vários tagmatas do corpo principalmente cabeça, abdômen e pernas do artrópodes (Jackson & Morgan 1993). As glândulas podem ser classificadas em glândulas endócrinas e exócrinas. As glândulas endócrinas destinam suas secreções (hormônios, por exemplo) na hemolinfa. E as glândulas exócrinas destinam sua secreção em cavidades do corpo (cavidade oral, por exemplo) ou em superfícies externas (cutícula, por exemplo) (LaFont 2000, Betz 2010). As glândulas exócrinas podem ser unicelular ou multicelular. No primeiro caso, uma única célula apresenta natureza secretória. Nas multicelulares, um conjunto de células (epidérmicas, dérmicas) são organizadas a fim de realizar a atividade secretora da glândula (Jackson e Morgan 1993). Glândulas exócrinas de insetos são historicamente classificadas em 3 classes, tendo como características a disposição da cutícula e a maneira como a secreção sai da estrutura (Noirot e Quennedey 1974). Em glândulas da classe 1, a célula é coberta pela cutícula e a secreção necessita atravessar essa barreira. A cutícula acima da glândula foi secretada pela própria glândula e está em contato com a célula da glândula. Em

glândulas classe 2, a célula da glândula é envolta por células epidérmicas mais ou menos diferenciadas. A cutícula acima da glândula não foi sintetizada e nem está em contato com a célula da glândula. Em glândulas da classe 3, um ducto ou canal cuticular penetra na célula da glândula e o canal é direcionado para um ducto ou canal cuticular celular secretado pela célula da glândula. Esse canal é contínuo com a cutícula e pode existir outra célula entre a célula glandular e o canal celular. Assim como na classe 2, a cutícula acima da glândula não foi sintetizada e nem está em contato com a célula da glândula (Noirot e Quennedey 1974, 1991). Vale ressaltar que uma glândula pode ser composta de uma ou mais classes de células glandulares e/ou outras células associadas.

Células glandulares apresentam estruturas internas que atuam em conjunto para o funcionamento da célula. Essas estruturas incluem retículo endoplasmático, complexo de golgi, ribossomos livres, vesículas de grânulos de secreção. Através do conhecimento dessas estruturas é possível indicar quais os principais compostos produzidos em uma determinada célula. Esses compostos podem secreções proteicas ou não-proteináceas (carboidratos e lipídeos) (Hopkins 1992, Betz 2010).

Compostos glandulares

Os invertebrados apresentam uma enorme diversidade de glândulas com uma enorme variedade de funções biológicas. Podemos classificar os compostos produzidos por glândulas de acordo com o principal produto da secreção. Os principais compostos são compostos alifáticos, fenóis, isoprenóides, compostos heterocíclicos, carboidratos, lipídios, aminoácidos, peptídeos e proteínas. Em muitos casos, a secreção de uma proteína não possui poucos componentes, mas são misturas estruturais e químicas altamente complexas. E determinados estudos (proteômica, peptidômica, cromatografia, espectrometria etc) são feitos para caracterizar e compreender as funções (LaFont 2000, Betz 2010, Scieuzo et al 2021).

Proteínas e peptídeos antimicrobianas

Os artrópodes tiveram sucesso em ocupar diversos nichos ecológicos e são constantemente expostos a diversos inimigos naturais, incluindo predadores e outros

potencialmente patogênicos. Para sobreviver, eles desenvolveram eficientes sistemas de defesa incluindo a cutícula, glândulas repugnantes, glândulas de veneno, sistema digestório, células da hemolinfa, proteínas e peptídeos antimicrobianos (Bulet et al 1999, Wu et al 2018).

O sistema imunológico dos artrópodes pode ser dividido em reações celulares e reações hormonais. Nas reações celulares ocorrem os processos de fagocitose, encapsulação ou formação de nódulos. Nesses processos estão incluídos a atuação de células apoptóticas, remodelação de tecidos danificados e o aprisionamento de microrganismos. Na reações humorais ocorrem os processos melanização, coagulação e síntese e/ou liberação de proteínas e peptídeos antimicrobianos (PAMs). Após uma injúria ou invasão da cutícula por microrganismos as células da hemolinfa (hemócitos) migram para o local de infeção. Esse processo pode resultar em fagocitose, nodulação ou encapsulação. Pode ocorrer também uma cascata de coagulação ou a liberação de moléculas antimicrobianas (Bachère et al 2004, Bulet 2004, Wu et al 2018).

Hidrocarbonetos cuticulares

A cutícula externa dos artrópodes é coberta por uma camada de cera composta por misturas de lípidos hidrofóbicos. Essa mistura inclui ácidos graxos, ésteres de cera, álcoois, aldeídos, esteróis e hidrocarbonetos cuticulares (HCs). Os HCs incluem alcanos de cadeia longa, alcanos ramificados e alcenos. O tamanho de uma cadeia de hidrocarbonetos varia de aproximadamente 20 a 50 átomos de carbono, como evidenciado pela cromatografia gasosa (Blomquist e Dillwith 1985). A primeira função conhecida dessa camada é manter o balanço de água e prevenir a dissecação do organismo. Outra importante função desses compostos cuticulares está relacionada à comunicação química de diversos animais, servindo como sinais químicos de contato (feromônios e cairomônios) (Millar 2010). Hidrocarbonetos cuticulares por exemplos, possuem papel no reconhecimento espécies e sexo durante interações reprodutivas, reconhecimento de parceiros de ninho, inimigos, status reprodutivo entre outros. Dessa forma, HCs são mediadores essenciais no comportamento dos artrópodes (Liebig 2010, Blomquist e Bagnères 2010).

O presente trabalho tem o intuito de explorar aspectos químicos e morfológicos de glândulas no grupo dos artrópodes, utilizando a espécie de opilião *Mischonyx squalidus* (Arachnida) como modelo de estudo. O trabalho é proposto em três frentes: A primeira tem por objetivo a caracterização morfológica interna e externa da glândula da membrana artrodial. A segunda tem por objetivo a caracterização química das proteínas da glândula da membrana artrodial bem como avaliar se algumas destas moléculas atuam de maneira bioativa contra microorganismos. E a terceira se propõe a caracterização química dos componentes cuticulares.

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INTRODUÇÃO ESPECÍFICA

Opiliões é um dos grupos mais diversos dentro da classe dos aracnídeos, com aproximadamente 6700 espécies descritas (Kury et al. 2020). Eles são divididos em 4 subordens: Ciphophitalmi, Eupnoi, Dyspnoi e Laniatores. Eles vivem em diversos habitats e apresentam hábitos noturnos em sua maioria (Curtis and Machado 2007). Um estudo morfológico descritivo de uma glândula na região da membrana artrodial da perna IV foi feito utilizando técnicas microscópicas e histológicas com a espécie *Mischonyx squalidus* (Capítulo 1). Além disso, para avaliar as propriedades moleculares presentes na estrutura glandular da membrana, um estudo proteômico foi realizado utilizando a secreção da estrutura (Capítulo 2). Após obtermos frações líquidas do conteúdo da estrutura glandular, testamos a existência e efetividade de moléculas bioativas frente a microrganismos (Capítulo 3). Por fim, para conhecer um pouco mais sobre compostos químicos encontrados na

cutícula dos opiliões descrevemos um conjunto de hidrocarbonetos cuticulares também em *M. squalidus* via cromatografia gasosa.

A existência de uma estrutura glandular na região da membrana artrodial não previamente descrita no grupo dos opiliões foi documentada, juntamente com o conjunto de moléculas (proteicas, por exemplo) produzidas nesta estrutura. Em adição, detectamos que a secreção glandular liberada da região da membrana artrodial de *M. squalidus* apresenta moléculas com atividade antimicrobiana. Por fim, encontramos diversos hidrocarbonetos cuticulares presentes em machos e fêmeas de *M. squalidus* que resultaram em apenas uma molécula diferente para as fêmeas.

As informações obtidas neste trabalho representam um avanço no conhecimento morfológico e químico em opiliões. Fornece informações sobre o funcionamento de estruturas glandulares em cutículas membranosas e o uso de seu produto como fonte de moléculas proteicas e outras moléculas biologicamente ativas (Silva et al 2021, 2023 aceito para publicação). Bem como trás a primeira descrição de diferenças em compostos cuticulares que podem estar associados a comunicação química no grupo dos opiliões.

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CAPÍTULO 1

Morphology of the arthrodial membrane gland in a Neotropical harvester (Arachnida, Opiliones)

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Morphology of the arthrodial membrane gland in a Neotropical harvester (Arachnida, Opiliones)

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Running head: Morphology of a gland in Opiliones

Abstract. We describe a gland in the arthrodial membrane of the coxa-trochanter articulation in the fourth pair of legs in the Neotropical harvester *Mischonyx squalidus* Roewer 1913. Externally the glandular area has a rough appearance with pores on its surface, with folds of the arthrodial membrane. Internally, its secretory cells have

spherical secretory vesicles, smooth endoplasmic reticulum, mitochondria and ducts that exit from the cells and cross the arthrodial membrane. Histochemical tests indicate the presence of proteins and neutral glycoproteins. The function of the gland might be to produce lubricating products that allow better movement of the coxa-trochanter region.

Keywords - elastic membrane, intersegmental membrane, lubrication, harvestman

INTRODUCTION

The body of arthropods is composed of two main types of cuticles, one more rigid and one softer (Hepburn 1976; Andersen et al. 1995, Moussian 2013). The often more rigid one is largely composed of exocuticle (ie, highly tanned) and forms the main plates (sclerites) and limbs of the skeleton. The softer cuticle makes up the flexible joints (intersegmental membrane) between the rigid parts and is composed almost entirely of hydrated endocuticle and epicuticle (Vincent 2002). An exocuticle is absent (Dennel and Malek 1954). Intersegmental membranes may be part of the joints of chela, head-thorax and legs among other body regions (Govindarajan et al. 1974, Tychsen and Vincent 1976, Andersen 1999). These structures have elastic properties that allow appendages to move properly (Hepburn 1976) and the protein resilin is probably responsible for flexibility (Bennet-Clark and Lucey 1967, Govindarajan et al. 1974, Neff et al. 2000, Gorb 2002). Histological studies in insects show that a specific intersegmental membrane called arthrodial membrane (= cuticle membrane – Gorb 1996) revealed exocrine glands (Billen 2009, Nijs and Billen 2015).

Studies with beetles and cockroaches have shown that they release lubricating substances in regions of the body that experience friction (femoro-tibial, head-prothorax, occipital region) (Naiden and Gorb 2021). These regions have pores (~0.5 μm – 10 μm in diameter) through which the lubricant comes out, usually in elongated cylindrical shapes, similar to toothpaste output. The authors suggest that the function of lubricants is to minimize friction and wear in the areas around these glands (Naiden et al. 2021, Naiden and Gorb 2021). Despite these recent discoveries, little is known about the internal morphology of membranes that make intense contact with other surfaces or themselves.

There is not a lot of information on arthrodial membranes in arachnids (see eg Shultz 2000; Sensening and Shultz 2003, 2004; Silva et al 2021; Schmidt et al 2022). With the aid of scanning microscopic images (SEM), Willemart et al 2007 found pores in the external region of the arthrodial membrane (plate pore) of the fourth pair of leg of two harvester (Opiliones) species, suggesting the presence of glandular structures (Arachnida, Opiliones). However, no previous studies have described the ultrastructure of the arthrodial membrane in this group. Harvesters have approximately 6700 described species (Kury et al. 2020). They inhabit preferentially humid areas, where they shelter in caves, under rocks, trees and logs (Curtis and Machado 2007) and are an interesting group of arthropods to investigate the structure and possible role of glands in intersegmental membrane regions. Thus, the objective of this work was to characterize the arthrodial membrane of the harvester *Mischonyx squalidus* based on light and electron microscopy (both transmission and scanning). Specifically, we asked whether we would find characteristics of glandular structures.

MATERIAL AND METHODS

Study species, collection and laboratory conditions

Mischonyx squalidus (Roewer, 1913) appears in previous articles as *Mischonyx cuspidatus* or *Ilhaia cuspidata* (see Gueratto et al. 2021). Individuals of *M. squalidus* (n = 10) were collected manually in August 2018 and March 2019, under tree trunks at the Parque Ecológico do Tietê (-23.494587, -46.521383), São Paulo city, São Paulo State, Brazil. Only male individuals were used because of their large arthrodial membrane. The animals were brought to the laboratory where they were supplied with water and dog food *ad libitum*. They were collected under SISBIO/ICMBio (Sistema de Autorização e Informação em Biodiversidade/Instituto Chico Mendes de Conservação da Biodiversidade) license number 61431-1- 2018. The term “canal” instead of “duct” or “channel” follows the classic paper of Noirot and Quennedey (1974) and several others that followed (eg Blomquist and Bagnères 2010; Kheyri et al 2014).

External Ultrastructure - Scanning Electron Microscopy (SEM)

To characterize the external morphology of the arthrodial membrane located in the coxa-trochanter joint of the leg IV of *M. squalidus*, the animals (n = 5) were first euthanized in a freezer at 4°C, then fixed in Bouin's solutions. The animals were submitted to three-step ultrasonic cleaning: (1) stirring with distilled water; (2) incubation with 1:10 detergent solution (Alconox); and (3) stirring with distilled water again. Then, the regions of interest were cut with micro scissors and dehydrated in graded series of 50% to 100% ethanol. Samples were then critical point dried and mounted on aluminum stubs with carbon adhesives on both sides. Finally, the samples were sputter coated with gold and photographed with SEM (Quanta 250, FEI Company, Netherlands).

Histological Anatomy - Histology and Light Microscopy

To reconstruct the internal morphology of the arthrodial membrane of *M. squalidus*, we anesthetized 5 individuals and fixed them in Karnovsky's (1965) or Bouin´s solution for 3 days. Subsequently, we cut the arthrodial membrane area with a micro scissor and embedded the samples in Leica Historesin. The samples were sectioned with a Microm HM 340 microtome 3 µm-thick. The histological sections were stained with hematoxylin and eosin. To characterize the chemical composition of the arthrodial membrane, we applied the following histochemical staining methods: bromophenol blue (for proteins), alcian blue pH 2.5 and periodic acid-Schiff (PAS) (for acid and basic mucopolysaccharides, respectively) and Sudan black B (for lipids). The samples were analyzed using light microscope (Leica) and photographed with a camera (Olympus) mounted on to the microscope.

Internal ultrastructure - Transmission Electron Microscopy (TEM)

To reconstruct the internal ultrastructure of the arthrodial membrane of *M. squalidus*, the samples were dissected in cold $NaH_2PO_4x2H_2O$ buffer and then fixed in a mixture of 2.5% Glutaraldehyde and 2% Paraformaldehyde in 0.1M buffer $NaH_2PO_4x2H_2O$ (according to Karnovsky 1965) and kept at 4°C. The samples were washed in Siena PBS buffer, fixed in 2% osmium tetroxide for 3 hours and washed again in Siena PBS buffer. For resin embedding (Spurr 1969) the samples were serially dehydrated from 50% to 100% pure ethanol gradually infiltrated in resin with alcohol/resin series 2/1, 1/1, 1/3 and pure resin. We made cross sections with 50 nm

thickness using an ultramicrotome (Microm HM 340). The samples were stained with uranyl acetate and lead citrate and analyzed (10 kV) in a Jeol JEM-100 CX22 Transmission Electron Microscope (TEM).

RESULTS

Arthrodial membrane: External structure, histological anatomy and internal ultrastructure

Externally, the arthrodial membrane of *M. squalidus* is located in the coxa-trochanter region of the leg IV (Fig. 1A) has smooth and textured regions of cuticle, which have several folds (“Fo”, Fig. 1B). Furthermore, there are pores of approximately 2 μm diameter dispersed on both surfaces (white arrows, Fig. 1B-D).

The arthrodial membrane (AM) is between the coxa (CX) and trochanter (TR), regions filled with muscles (m) responsible for leg movements, and it stains more clearly with hematoxylin in the histological sections (Fig. 2A, B). The AM is clearly thinner than regions with cuticle only (Fig. 2B), and extends the whole circumference of the trochanter although it is visible only on the dorsal region in the figure (Fig. 2B). Folds composed of a stratified fibrous matrix can be observed (Fig. 3A-C). The epidermal region is composed of several secretory cells with a prismatic shape, found exclusively in arthrodial membrane regions (Fig. 3). Each cell has a nucleus arranged in the center and the cytoplasm is filled with granules of varying shapes (Fig. 3C). Irregular cuticular canals can be observed within the cells and arthrodial membrane (Fig. 3B, C).

The histochemical analysis showed that the arthrodial membrane reacts only with Bromophenol Blue and PAS, indicating the presence of proteins (Fig. 4A) and neutral glycoproteins (Fig. 4B). It was not possible to identify the reaction of Bromophenol Blue and PAS in the prismatic cells because some cells were damaged at some point during histological procedures. Through the histochemically treated images, it was also possible to observe irregular cuticular canals penetrated in the prismatic cells and in the arthrodial membrane. (Fig. 4 A,B). We found no reservoir in this set of cells. It is hard to be exclude the possibility that the hundreds of small dark spots stained granules are not an artifact, and therefore we cannot be sure if they correspond the observed granules in TEM.

The TEM images also provide evidence that prismatic cells are full of secretion granules with variable morphology and electron density (Fig. 5A,B). The electron-dense granules have a diameter between 0.4 and 0.8 μm. Also, a smooth endoplasmic reticulum (SER) could also be observed (Fig. 5A) in the cytoplasm of cells, between the secretion granules, in addition to mitochondria (Fig. 5B).

DISCUSSION

Our study demonstrates that the arthrodial membrane of the coxa-trochanter joint of leg IV of a harvester has cuticular pores in the outer region and cuticular canals internally. The arthrodial membrane presents also proteins and glycoproteins as well as a set of prismatic cells in the epidermal region.

The external rough appearance of the arthrodial membrane outside the pore plate may be related to elastic properties of the membrane. Not only do the SEM micrographs show folded areas but the histological analysis also shows contracted or folded cuticle. Indeed, typically arthrodial membranes differs from adjacent cuticle (see eg Hackman 1982). We also found that the arthrodial membrane consists of, among other components, proteins and glycoproteins. The lower degree or absence of sclerotization combined with specific proteins give elastic properties to the arthrodial membranes of many arthropods (Willis 1987, Andersen 1999, Moussian 2013). A protein with elastic proteins commonly found in arthropods including harvesters is resilin, which efficiently stores elastic energy. Though resilin fluoresces blue under UV light (Michels et al. 2016), as did AM (NFS Silva, personal observations), we do not have evidence of its presence. Folds similar to the reported herein have been previously reported (Compere and Goffinet 1987), and variations of such folds are common in regions of soft cuticle in insects (Hackman and Goldberb 1987).Microscopically, the external surface of the arthrodial membrane has several cuticular pores, which suggested the presence of canals to release secreted material. Canals have been previously reported in the AM (Billen 2009; Billen and Plancken 2014; Nijs and Billen 2015) and, indeed, our histological section revealed that the prismatic cells below the arthrodial membrane have cuticular canals that run from the secretory cells to the membrane itself (Fig. 3B,C and 4A,B). The irregular nature of canals has also been observed in previous studies (Hackman and Goldberg 1987). The cuticular canals we found were probably filled with secreted material (Fig. 5B),

which suggests that the glandular material must exit to the outer region of the arthrodial membrane. However, it was not possible to find a relationship between the amount of cuticular canals and the secretory cells observed. A fibrous matrix similar to the found here and cells with mitochondria have been reported in arthrodial membranes of insects, but not with such prismatic cells (Grandperrin and Cassier 1983; Hackman and Goldberg 1987).

The composition of the secretion granules showed differences in electro densities. That could be related to the degree of maturation of these granules (though we cannot discard the possibility that these are granules of different substances), and be evidence of secretory activity in the specimen studied (Sobotnik et al. 2003). Moreover, the shape of these granules suggests their composition include proteins (Sobotnik et al. 2003, Billen 2009). This would be in agreement with a study on beetles, where the semi-solid lubricating substance is mostly insoluble protein (Naiden et al. 2021). Considering that harvesters joints are exposed to the environment, insoluble proteins allow that they will not be lost in contact with humidity, which is an important feature for harvester living in humid regions (see Curtis and Machado 2007). It should be mentioned that Silva et al (2021) have reported proteins related to transport, lysis, storage of lipids and with possible antimicrobial function in this exact gland this harvester species. Such proteins may also be related to the vesicles described herein. Finally, the presence of mitochondria suggests some secretory activity in these cells.

The works that described exocrine glands (histological) in intersegmental membrane regions are almost exclusively carried out in insects, mainly ants and wasps (Sobotnik et al. 2003, Billen 2009, Nijs and Bilen 2015). The coxal-gland was described in the same region of the arthrodial membrane between the coxa-trochanter of harvesters (Willemart et al 2007, Billen and Vander-Plancken 2014, Nijs and Billen 2015). This gland differs from ours in being a bicellular gland (class-3, see Noirot and Quennedey 1991) while that of *M. squalidus* has only one cell unit (probably class-1, Noirot and Quennedey 1991). The other works described several other exocrine glands, mostly class-3 secretory cells, belonging to other regions of the intersegmental membrane. Almost unanimously, the authors suggest that these glands have a lubricating function. The main reasons for this conclusion, which also apply to the species we have studied, are the presence of the gland close

to articulation regions, as well as canals that open close or properly in intersegmental articulation membranes such as trochanter-femur, distal femur, proximal and distal junction of tarsomeres, femur-tibia, proximal and distal tibia, coxa-thorax (Schoeters and Billen 1993, Billen 2009, Billen and Vander-Plancken 2014, Nijs and Bilen 2015). Other possible reasons that likely also apply to harvesters (though we have not tested) are the presence of these glands in all legs, and in in both sexes, the repetition of these glands in segments (such as the tarsomeres) and, finally, the presence of organelles such as a smooth endoplasmatic reticulum (Schoeters and Billen 1993, Billen 2009, Billen and Vander-Plancken 2014, Nijs and Billen 2015). It has been shown that lubricants prevent physical contact between surfaces, absorbs energy and minimizes friction and wear (Naiden et al. 2021).

Because other joints of harvesters also have similar morphologies including pore openings, they possibly also have intersegmental glands (NFS Silva, personal observation). Based on the several suggestions found in the literature, such as gland location, pore opening site, proteins substances, presence of organelle with possible indication of production of oily substances (Attygalle et al. 1996, Billen and Ito 2006, Billen 2009) and exclusivity of the prismatic cells in the arthrodial membrane, we also suggest that the cells of the epidermal region of the arthrodial membrane of *M. squalidus* may be a gland with a lubricating function. Based on the similarities in the morphology of other intersegmental regions and joints to the one studied, such function would also be present in other body regions in harvesters.

We provided a first structural characterization and described general patterns of the chemicals present in an arthrodial membrane in Opiliones. Evidences from both areas suggest a secretory function, with the function to be determined

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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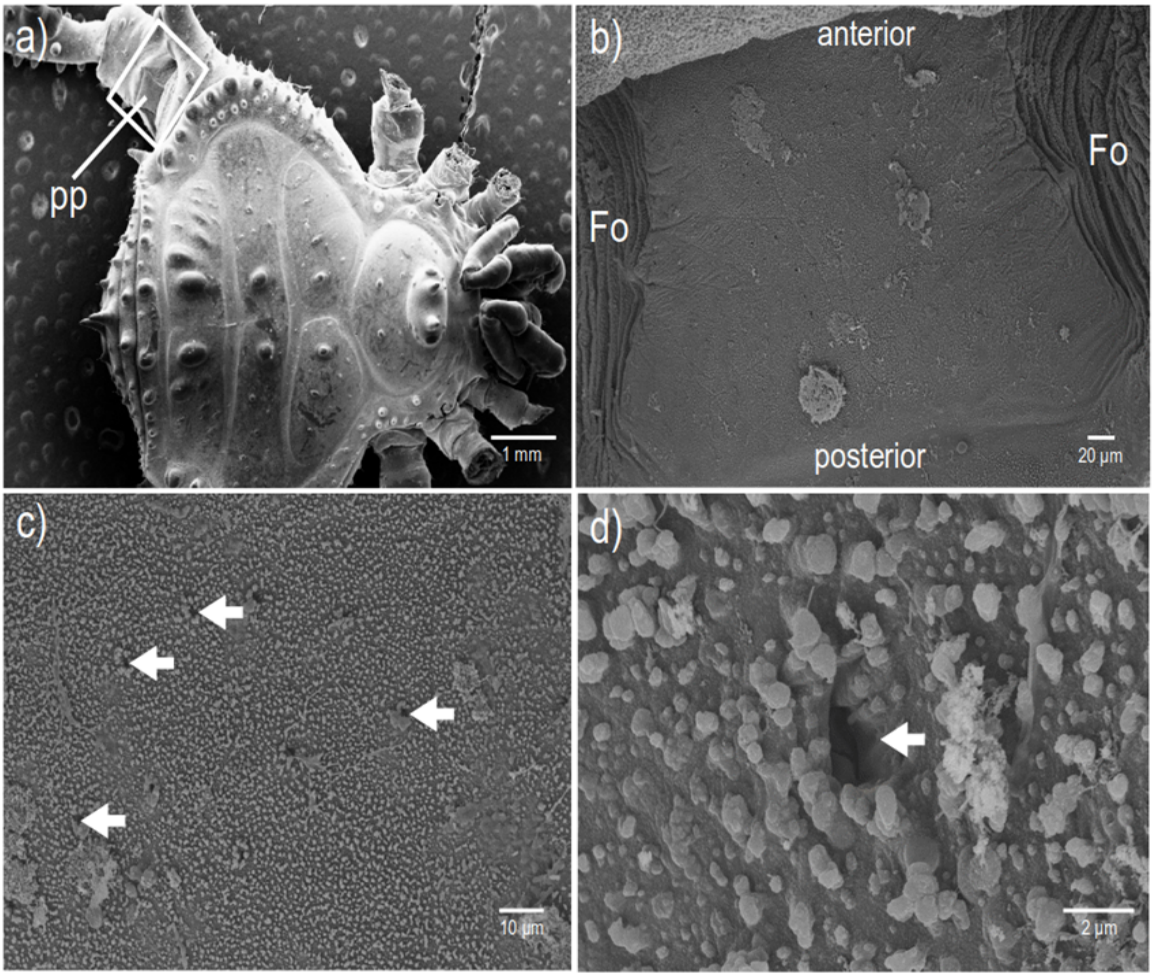

Figure 1. External morphology of a male harvester *Mischonyx squalidus* (Arachnida, Opiliones). **A**. Dorsal view. The anterior region is on the right, legs I, II and III were removed. The square shows the arthrodial membrane in the leg IV and the pore plate (pp); **B**, **C** and **D** show increasing zoom of the pore plate, a region without folds. **B** Regions with folds (Fo) and without folds. White arrows show pores.

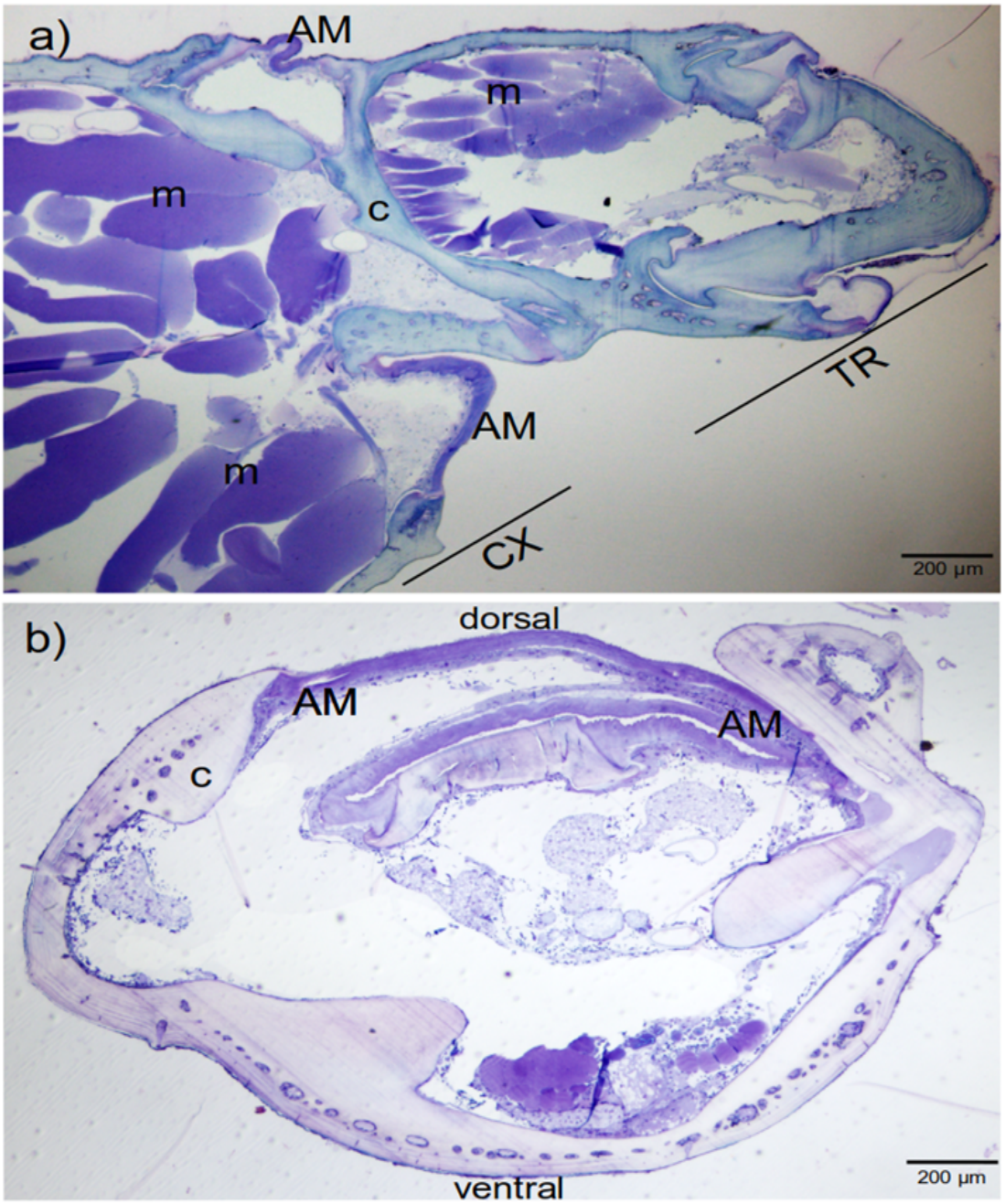

Figure 2. Sections through an arthrodial membrane of the coxa (CX) - trochanter (TR) articulation of a leg IV in a male harvester *Mischonyx squalidus* (Arachnida, Opiliones). **A**. Frontal longitudinal section. **B**. Transversal section between the coxa and the trochanter of leg IV. AM = arthrodial membrane; c = cuticle (sclerite cuticle); m = muscle.

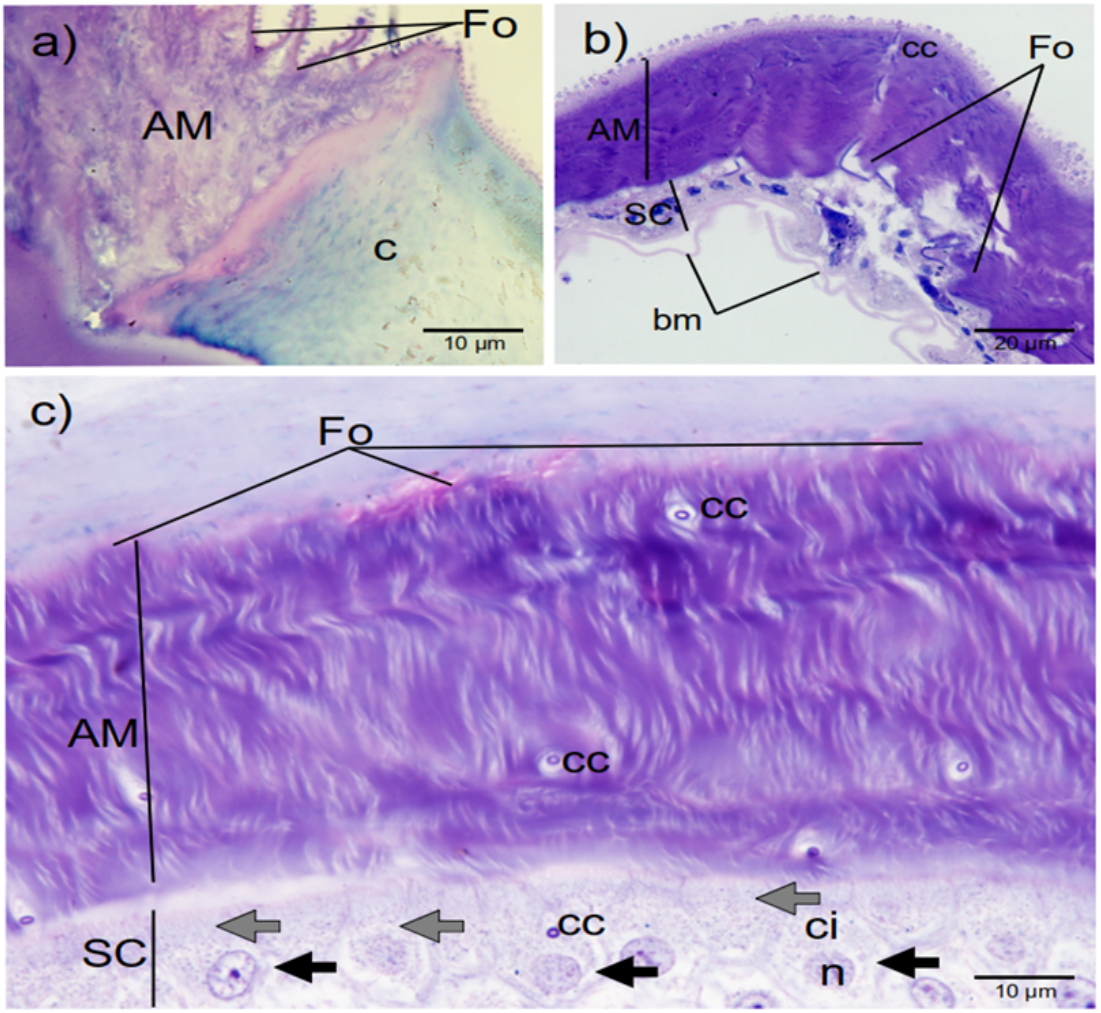

Figure 3. Sagital sections through an arthrodial membrane of the coxa – trochanter articulation of a leg IV in a male harvester *Mischonyx squalidus* (Arachnida, Opiliones). **A**. Arthrodial membrane cuticle (AM) and cuticle (sclerite cuticle) (c). **B**. Arthrodial membrane and basal membrane of secretory cells. **C**. Secretory cells (sc) with glandular prismatic cells (black arrows), granules (gray arrows) and cuticular canals (cc) stained with hematoxylin and eosin. bm = basal membrane; ci = cytoplasm; Fo = folds; n = nucleus.

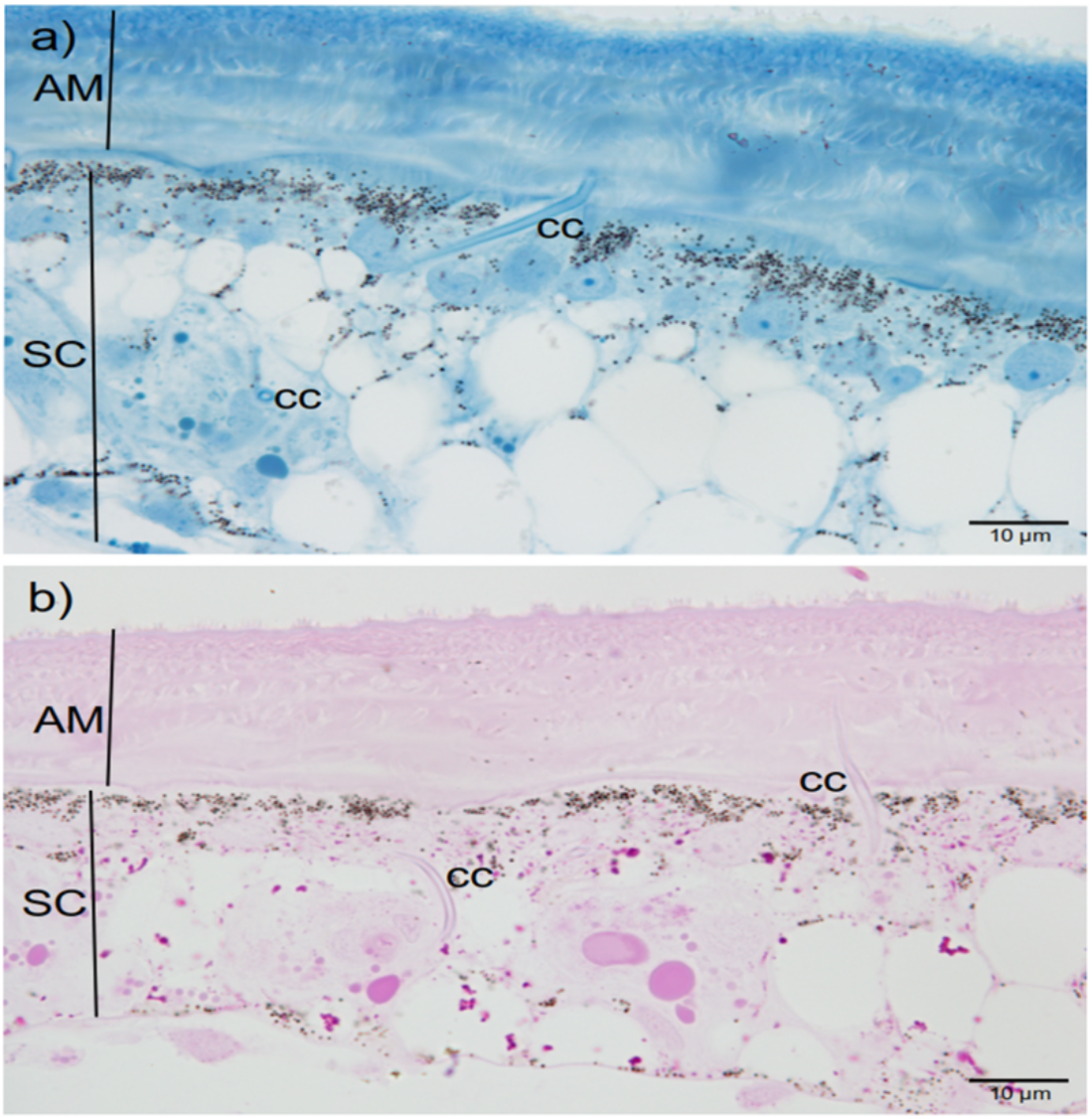

Figure 4. Sagital sections through an arthrodial membrane of the coxa – trochanter articulation of a leg IV in a male harvester *Mischonyx squalidus* (Arachnida, Opiliones). **A**. Staining with bromophenol blue. **B** Staining with PAS. AM = arthrodial membrane; SC = secretory cells; cc = cuticular canals.

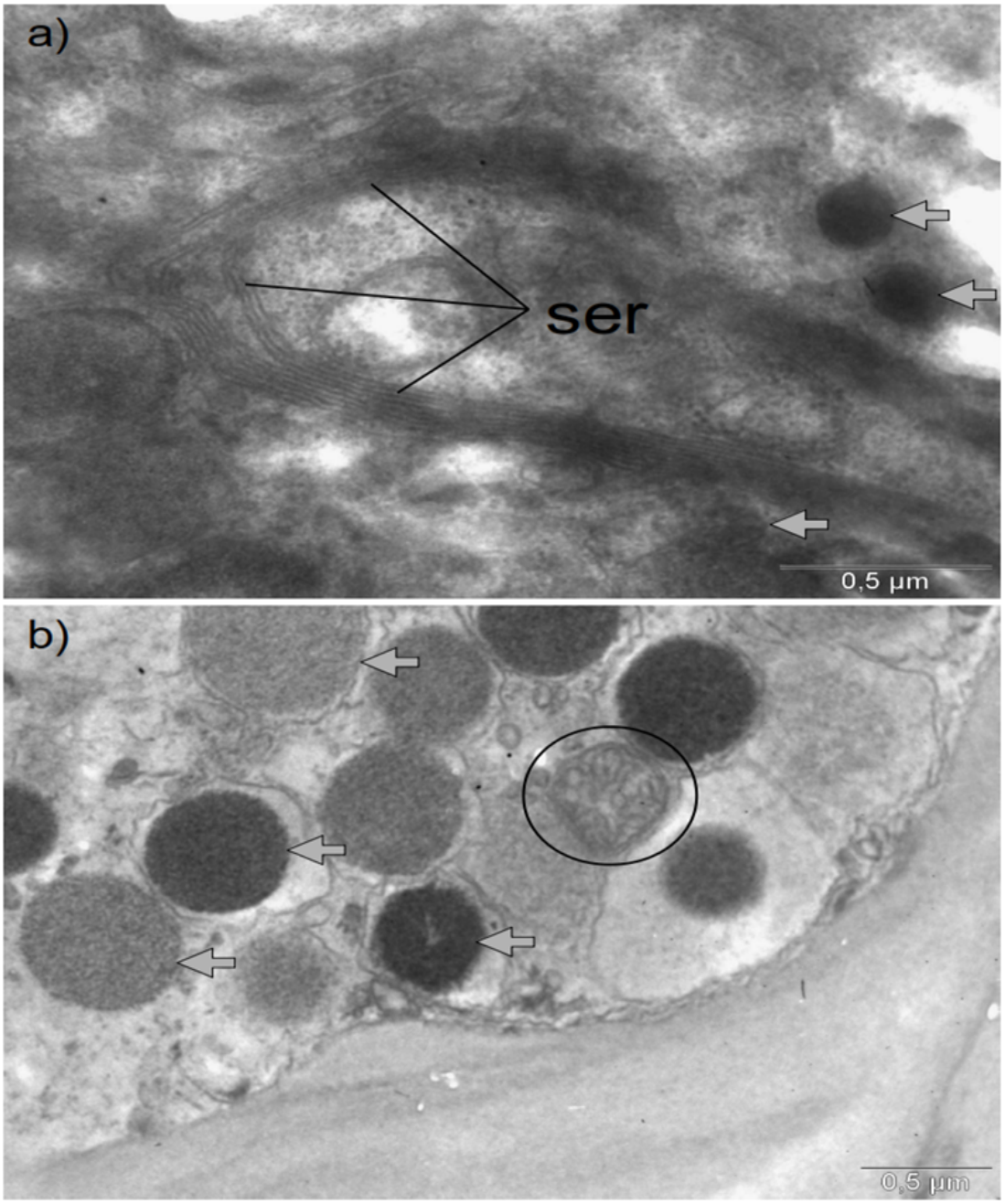

Figure 5. Interior of a prismatic cell in the arthrodial membrane of the coxa – trochanter articulation of a leg IV in a male harvester *Mischonyx squalidus* (Arachnida, Opiliones). **A**. Smooth endoplasmic reticulum (ser). **B**. Mitochondrion (circle) and granules (gray arrows).

CAPÍTULO 2

Protein components of the arthrodial membrane gland in a Neotropical harvestman (Arachnida, Opiliones)

Protein components of the arthrodial membrane gland in a Neotropical harvestman (Arachnida, Opiliones)

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ABSTRACT

The content of arthrodial membrane glands in arthropods has seldom been studied. Here, we have analyzed the proteins of the arthrodial membrane gland of the trochanter-coxa articulation of the fourth pair of legs in the harvestman *Mischonyx cuspidatus* via reverse-phase high performance liquid chromatography (RP-HPLC), poliacrylamide gel electrophoresis (PAGE) and nano-scale liquid chromatography coupled to mass spectrometry (nLC-MS/MS) analysis. Most of the fractions studied are hydrophobic, being proteins with molecular weights of ~28KDa, 62 KDa and ~198 KDa. These proteins seem to be homologous to proteins involved in product secretion, cytoskeleton, protein binding, cellular metabolism and antimicrobial action among others. A lubricant function is also possible based on the literature. We were able to identify 147 proteins in the inner region, 89 proteins from the outer dorsal region and 36 proteins from the outer ventral regions. Some proteins are present only in one of these regions, and some are shared by one or more regions. Our work provides, to the best of our knowledge, the first proteome characterization of the content of an arthrodial membrane gland in arachnids. Dataset Identifier: ftp://massive.ucsd.edu/MSV000087195/

Keywords: peptide, proteomic, arthrodial membrane gland, arachnida, opiliones

INTRODUCTION

The body of arthropods is covered by thicker and thinner cuticle depending on the region.[1] The thicker cuticle typically covers most of the body except few areas such as the junction between the body parts.[2,3] These junctions are known as arthrodial membrane, which is a soft and flexible cuticle with elastic properties.[4] Below the arthrodial membrane of some insects, glands thought to have lubricating function have been found.[5,6] These glands have ducts that open at the arthrodial membrane and probably work as the outlet for the secretion for the outer body of the organism.[5,6] It has been shown that the arthrodial membrane of a lobster is composed of water and a small amount of chitin–protein fibers. However, there is no work reporting the chemical composition of arthrodial membrane glands.[7] It is suggested that these glands have a lubricating function that might be related to their

location in articulation regions in the body of animals such as the head-thorax, trochanter-femur, coxa-thorax etc.[5,6]

The arthrodial membrane is also known to be a site of worm colonization and invasion by fungi and bacteria.[8] Spiracles and arthrodial membranes of insects are used by parasites to invade a host body.[9,10] Therefore, the presence of glands per se is not enough information to allow conclusions on their function. A first step towards a functional understanding of the function of the gland is to study its content by identifying the group of molecules in the gland and compare them with molecules of known function in the literature. The following step would be to identify, isolate and further run bioassays to confirm its function.

Harvestmen belong to the class Arachnida and have approximately 6700 described species.[11] They typically inhabit humid areas where they shelter in caves, rocks, trees and fallen logs.[12] As in other arthropods, leg joints are separated by an arthrodial membrane. Scanning Electron Microscopy images of these areas have shown that the external region of the arthrodial membrane in harvestmen may bear dorsal pores of approximately 1 μm diameter.[13] Such pores suggest that harvestmen release glandular secretions,[13] but to the best of our knowledge there are no studies on the chemical composition of these secretions. In this work, we carried out the proteomic characterization of the secretion content found in the arthrodial membrane of a Neotropical species, *Mischonyx cuspidatus* Rower 1913 (Arachnida, Opiliones, Gonyleptidae), considering both the outer (dorsal and ventral) and inner contents of the arthrodial membrane gland. We expect that such description may help the understanding of the function of this gland.

MATERIALS AND METHODS

Study species and laboratory conditions

Individuals of *Mischonyx cuspidatus* were collected manually (in August 2018 and March 2019) under tree trunks at the Parque Ecológico do Tietê (-23.494587, -46.521383), São Paulo city, São Paulo State, Brazil. The animals were collected under SISBIO/ICMBio licence number 61431-1- 2018. We used only males because they have a larger arthrodial membrane gland that facilitates the collection of the secretion (Figs 1a and b). Harvestmen were fed with dog food and provided water *ad libitum*.

Extraction of glandular secretion

We first aimed to extract the inner secretion of the arthrodial membrane gland. After anesthetize the animals (n = 5) in a freezer at -20º C (15 min), we made a perforation in the arthrodial membrane using a micro needle and we collected the secretion (~15 µL) with an ultrafine syringe with 1 mL of ultrapure water (Seringa Insulina BD Ultra-Fine U-100, needle 6 mm). Water was used to dilute the secretion and the solution was placed in an eppendorf with another 1 mL of ultrapure water and stored in a freezer.

In order to compare the secretions of the inner region with the outer dorsal and ventral regions (Figs 1a and 1b), we rubbed clean cotton swabs on the dorsal and ventral outer regions of the gland. Cotton swabs from each region were placed in separate vials containing 50 mL of 50% acetronitrile (ACN, Sigma-Aldrich). We used one cotton swab per individual for the dorsal and one for the ventral region (n = 136 males).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to verify the protein profile of the secretions of the inner region of the arthrodial membrane gland, we ran the samples in a Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE,[14]). The extracts were stained with Coomassie-R blue. Twenty µg of pure extract of the inner secretion, solubilized in 20 µL of ultrapure water, with a sample buffer were injected in each lane. It was then submitted to electrophoresis under no reducing conditions on 15% SDS-polyacrylamide gel, using invitrogen SeeBlue (Life Technologies, São Paulo, Brazil) as a molecular weight marker. Since the extraction of the gland secretion involves the perforation of animal tissue, we used the hemolymph as a means of extraction control. After the animals (n = 5) were anesthetized at 4 ° C, we cut the patellar region of the leg IV and collected the hemolymph (~ 15 µL) with a micropipette. Hemolymph was analyzed on polyacrylamide gel under the same conditions as described for the inner secretion.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

Here our aim was to compare the chromatogram profile of three different regions of the gland, “inner”, outer “dorsal” and “ventral. RP-HPLC analysis was performed in a 60 min gradient at a 1.0 mL/min flow rate. We ran RP-HPLC separation with a C18 column (Jupiter, 4.6 mm × 250 mm) equilibrated with 0.05% TFA. The elution gradient for the sample was 0-80% of solution B (0.05% (v/v) TFA in ACN) in solution A (0.05% (v/v) TFA in water). Effluent absorbance was monitored at 225 nm, and the fractions were hand collected, concentrated under vacuum, and reconstituted in ultrapure water. For both the inner and the outer regions (dorsal and ventral), we used a 10 µg/µL of secretion for the chromatograms.

Mass Spectrometry (LC/MS)

The fractions obtained on the RP-HPLC analysis were run into a nLC Easy (Thermo Fisher Scientific, Bremen, Germany) coupled to an LTQ XL Mass Spectrometer, (Thermo Fisher Scientific). The samples were diluted in 10 µL 0.1% formic acid (FA) prior to injection. The liquid chromatography was performed using a house prepared analytical and precolum C18 column as previously described[15]. A linear gradient from 5% to 80% of acetonitrile in 0.1% FA during 15 min at a 1uL/min flow. The spectrometer was set to a positive parameter.

Bioinformatics

In order to identify the molecules, the raw files generated by the LTQ-XL Mass Spectrometer were loaded in MASCOT Deamon R (Matrix Science, Inc., Boston, MA) version 5.4.2 software. Since there is no harvestman database in the Uniprot/Swissprot databank, we ran the search against the Arthropoda databank downloaded from Swiss-Prot in July 2019. We use the following parameters for the search: Enzyme = none; Variable modifications = “Oxidation (M)”; Fixed modifications = none; Mass values = Monoisotopic; Protein mass = Unrestricted; Protein and Peptide mass tolerance = “1 Da”; Significance threshold = $p<0.05$; Ions score or expected cut-off = -1. We use only predicted protein with score greater than or equal

to 40. This score was chosen as the cut-off between real hits and possible false positive results.

The peptides and proteins identified in the secretion of the arthrodial membrane gland of *M. cuspidatus* were further searched in the site UNIPROT for their biological function.

RESULTS

Protein profile of inner secretion of arthrodial membrane gland by SDS-PAGE

The non-reducing SDS-PAGE (15%) of raw extract of the inner secretion of arthrodial membrane gland resulted in several bands (Fig 2 and Figure S1). One group with molecular weight between ~62 kDa and ~198 kDa, and another group with molecular weight at ~28 kDa. The hemolymph fraction shows bands with low molecular mass at ~62 kDa and ~28 kDa.

Protein fractionation by (RP-HPLC)

The RP-HPLC analysis profile of the extracts of the inner and outer (both dorsal and ventral) secretions revealed some fractions (three to seven), hydrophilic and several in the hydrophobic fractions of each region (Fig 3). The profile of the dorsal and ventral chromatograms (Fig 3) showed the same profile as the inner region.

Mass Spectrometry and Bioinformatics

Mass spectrometry analysis of the inner and outer gland secretions allowed us to identify several peptide and protein fragments that are homologous to peptides and proteins described in the literature in other arthropod species (Table S1). We found 147 proteins sequences in the inner region, 91 proteins sequences on the dorsal region and 36 proteins sequences on the ventral region (Table S1 and S2). Many of the identified sequences are unique (support material: Dataset Identifier: ftp://massive.ucsd.edu/MSV000087195/; Figure S2, S3, S4 and S5).

By comparing the peptide and proteins (obtained via database) present in the inner and outer (in both dorsal and ventral regions), we observe that some molecules are

present in more than one region (Fig 4), a pattern that is also clear looking at the Venn diagram. Nine molecules are present in the inner and dorsal region, one molecule is present in the inner and ventral region and 2 molecule are present in the dorsal and ventral region. The diagram shows that several molecules are exclusive to one of the regions, however many molecules appeared more than once in the three regions and in different fractions obtained from the liquid chromatography fractionation (Table S3). The inner region of the gland presented 121 unique molecules, the dorsal region 57 molecules and the ventral region 27 molecules (Fig 4).

The analysis of peptide and protein functions of the identified molecules in the 3 regions of the gland (i = inner, d = dorsal and v = ventral) via UNIPROT (see Table S2), showed that they may be the product of glandular secretions, be structural, ribosomal, have a role in metabolism, defense against pathogens and signaling.

DISCUSSION

In this work we were able to identify a total of 274 molecules (proteins and/or peptides homologues) that we attribute to be accumulated in or produced by the arthrodial membrane gland of the coxa-trochanter region of *M. cuspidatus*. We found hydrophilic and hydrophobic molecules putatively homologous to peptides and/or proteins with functions such as cellular metabolism, signaling and binding, defense and microbial activity. In addition, we observed high similarities between the inner and the outer regions.

The SDS-PAGE of the extract of the inner secretion of arthrodial membrane gland resulted in two major groups of molecues with high (> 100 kDa) and low (62 kDa and ~28 kDa) molecular weight. The hemolymph which was used as a control of the extraction of glandular content had a low (~28 kDa) molecular weights, suggesting that the inner secretion and hemolymph are two different compounds. However, it is likely that there are similar compounds as glands are nourished by hemolymph[16].

Through RP-HPLC we found several hydrophobic fractions. Many fractions have similar retention times mainly between the outer regions (both dorsal and ventral).

For example, the fractions four and seven (Fig 3b and 3c) presented similar molecules with similar sequences (Table S3). These fractions probably correspond to the same peptides or proteins as evidenced by mass spectrometry.

In our search, all molecules identified by the mass spectrometry analysis matched to some extent peptides or proteins from the Arthropoda database. Many molecules were found in a unique region (inner or outer - both dorsal and ventral), but some were found in more than one region. We were able to identify a higher number of proteins in the inner region (147 proteins, of which 121 are unique to this region). In addition, we identified nine shared molecules in the inner region and one molecule in the dorsal region. The exact biological role of the arthrodial membrane gland is not yet known but we have evidence that the dorsal region has several pores that are likely to serve as outputs for these molecules (NFSS unpublished data)[13]. Cytochrome c oxidase subunit 1 protein is the only protein shared by the inner and ventral regions, but we do not know why this molecule is found outside the animal.

The existence of two molecules found exclusively on the ventral and dorsal regions may be the result of proteins cleaved from the inner region. Since the inner region has some enzymes (Table S1) it is possible that they act on protein cleavage, making them active and performing their functions in the outer region of the gland.[17,18]

We have found proteins that have been related to transport, lysis and storage of lipids (Table S1 and S2) and smooth endoplasmatic reticulum (NSF, unpublished data). These observations have also been reported previously in studies of the presence of secretory glands between joints of insect appendages.[19,20] Previous authors have suggested a lubricating function for the secretions.[5,6] If this is true, it would probably also apply to harvestmen in our study. In addition, we found similarities to 5 bioactive molecules (Protein diedel, U-poneritoxin, Ceratoxin-B, Tachystatin-A2) in the inner region and only one in the dorsal region (Peptide ctri9293). These molecules may have antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses and fungi[21,22,23,24] (Table S1 and S2). Since the arthrodial membrane region is possibly a site of colonization and possible invasion by pathogens[10], the similarity of molecules with others with antimicrobial activity indicates that the arthrodial membrane gland may have components necessary for the defense of the organism against infection. Finally, antimicrobial molecules are of

great medical importance, and since we have evidence that these molecules can be found in the structures of harvestman, this work contributes to a possible new source of bioactive molecules for new drug discovery[25].

CONCLUSION

We were able to identify and characterize possible peptides and proteins from the secretion of the *M. cuspidatus* arthrodial membrane gland and this study represents an important new data for this type of structure. To our knowledge, this is the first study to describe the composition of an arthrodial membrane gland in arachnids. This set of molecules may be used as a basis for future studies of the arthrodial membrane gland in Opiliones and other arthropods with similar structure.

AUTHOR CONTRIBUTIONS

Conceptualization, NFSS, RHW, PISJ, JRMCS; methodology, NFSS, RHW, PISJ, JRMCS; validation, NFSS; formal analysis, NFSS; investigation, NFSS; resources; data curation, NFSS; writing—original draft preparation, NFSS, RHW; writing—review and editing, NFSS, RHW; visualization, NFSS, RHW; supervision, RHW, PISJ, JRMCS; project administration, NFSS, PISJ, JRMCS; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPORTING INFORMATION

- List of proteins identified in *Mischonyx cuspidatus*.
- Functional classification of proteins found in *Mischonyx cuspidatus*.
- Sequences of some proteins identified in *Mischonyx cuspidatus*.
- Non-reducing SDS-PAGE of crude extract of the inner content of the arthrodal membrane gland.
- Mass spectrum of 5 proteins representing the studied proteins.
- Deconvolution plot, mass error and peptide sequence obtained from the main proteins (inner, dorsal and ventral) via MASCOT software.

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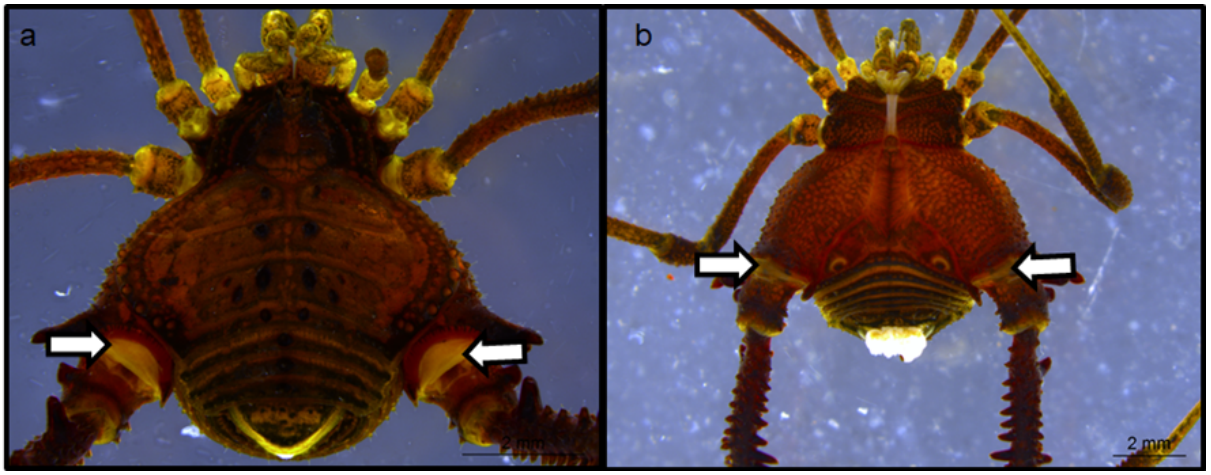

Figure 1. Male harvestman *Mischonyx cuspidatus* in (a) dorsal and (b) ventral views. Arrows indicate the arthrodial membrane of the region between the coxa-trochanter.

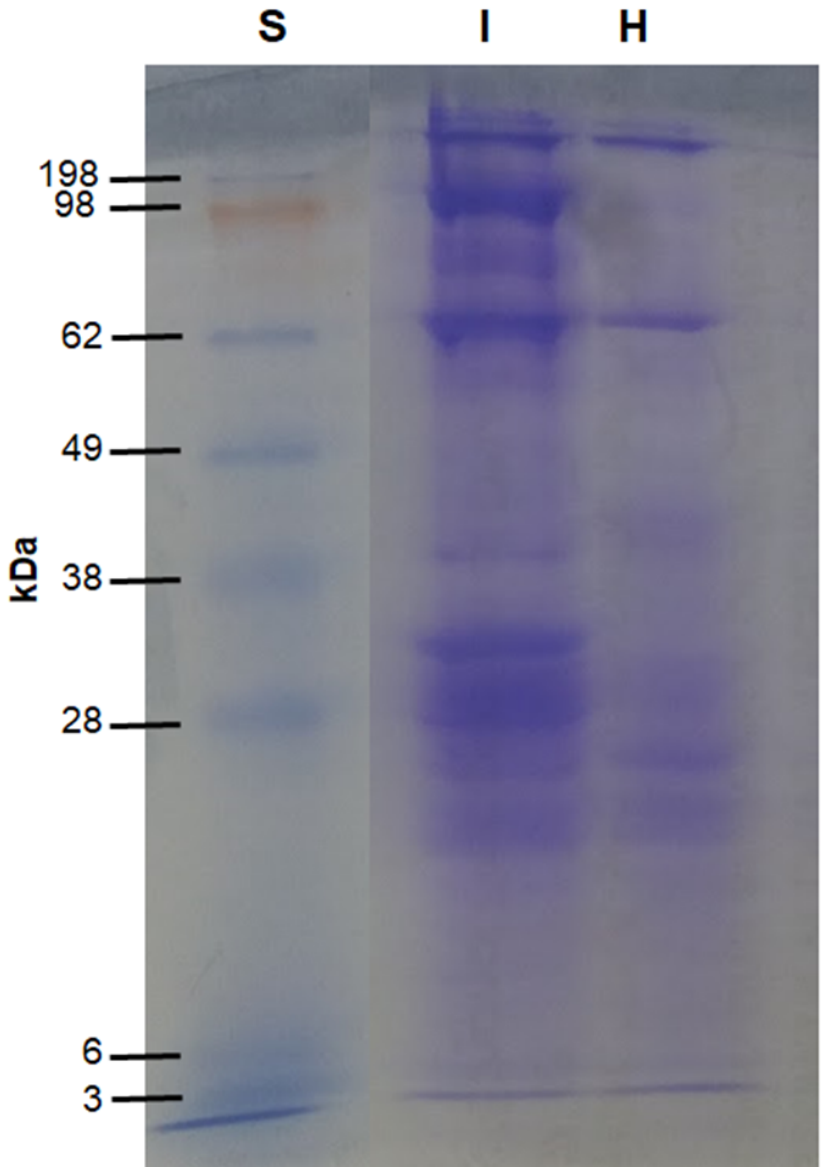

Figure 2. Non-reducing SDS-PAGE (15%) gel showing the protein profile from the standard stain (S) (left lane), the inner secretion of the arthrodial membrane gland (I) (middle lane) and hemolymph from the patella region (H) (right lane) of the harvestman *Mischonyx cuspidatus* male, (SeeBlue Plus2 Pre-stained Protein Standard, Invitrogen). Numbers at left indicate the mobility of molecular mass markers.

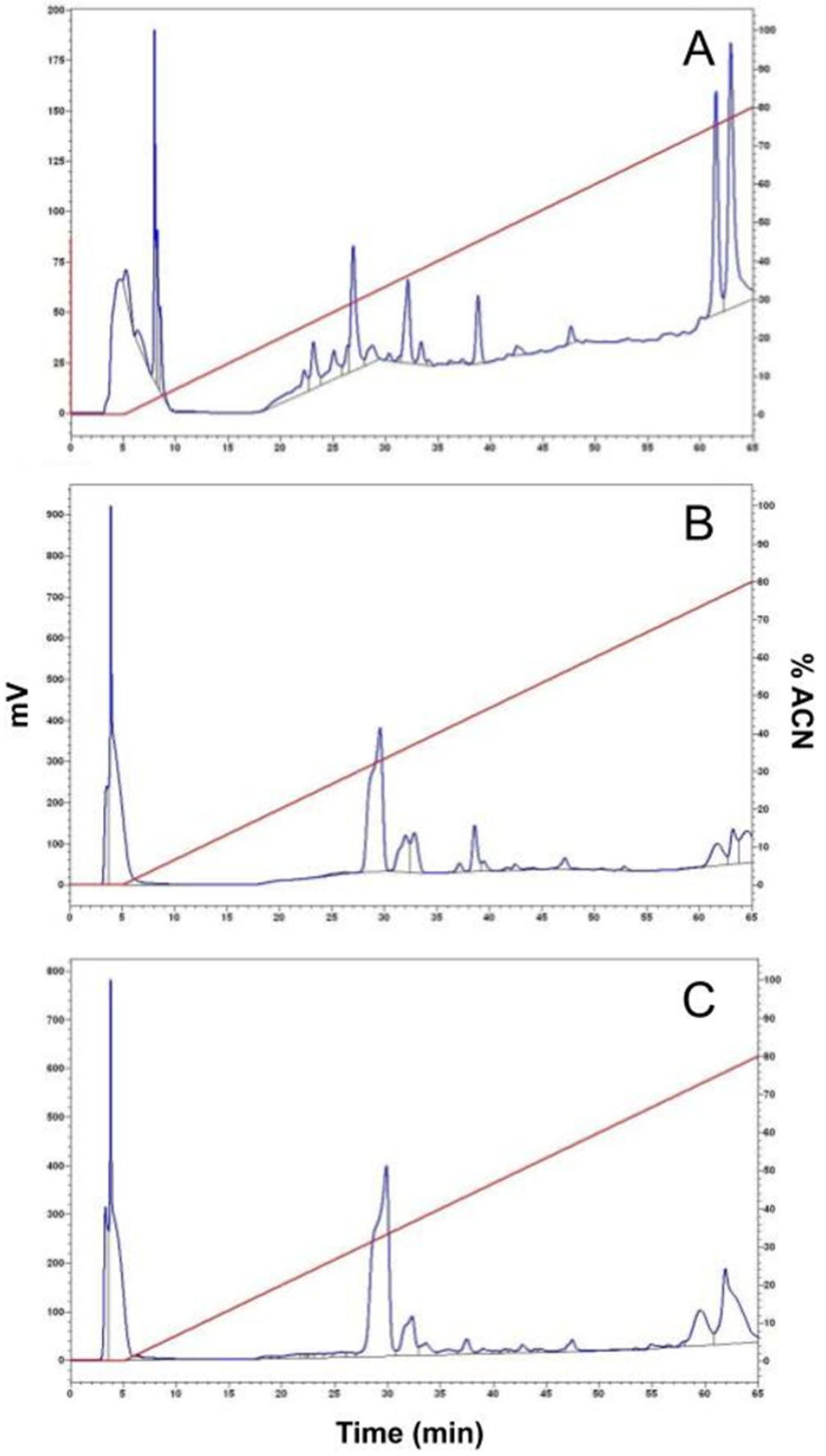

Figure 3. Chromatographic profile of the purification step of the secretion of the arthrodial membrane of the region between the coxa-trochanter in *Mischonyx cuspidatus*: (a) inner secretion, (b) dorsal (outer) and (c) ventral (outer) regions.

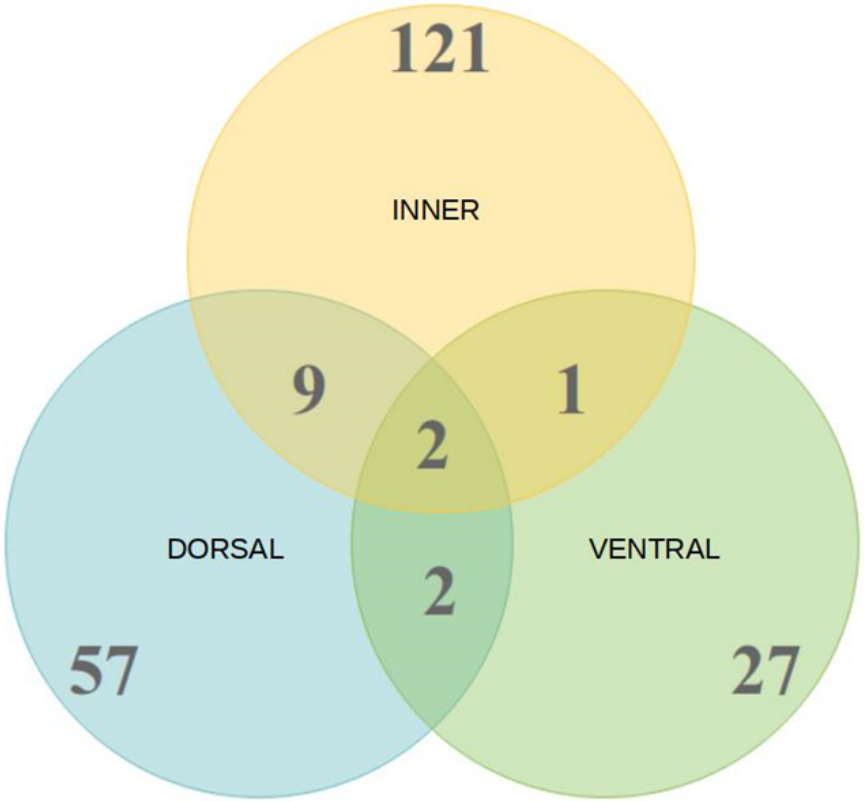

Figure 4. Venn diagram of the putative proteins of arthrodial membrane of the harvestman *Mischonyx cuspidatus*. The numbers indicate the quantity of proteins found in three regions of the arthrodial membrane. Inner: proteins found inside the gland; Dorsal (outer): proteins found on the dorsal region of the arthrodial membrane; Ventral (outer): proteins found on the ventral region of the arthrodial membrane.

CAPÍTULO 3

Molecules with antimicrobial activity in the secretion of the arthrodial membrane gland of a harvester (Arachnida, Opiliones)

Molecules with antimicrobial activity in the secretion of the arthrodial membrane gland of a harvester (Arachnida, Opiliones)

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Abstract.

Because of the exoskeleton, arthropods must have flexible areas in order to be able to move. Such regions are called arthrodial membranes and are particularly vulnerable to bacteria and fungi. Here we analyzed the secretion in the glands underneath it in a Neotropical harvester (Arachnida, Opiliones) and tested whether it has antiseptical properties. We punctured the membrane, collected and diluted the secretion and quantified proteins and peptides in a spectrophotometer. We also fractionated and analyzed the samples in a Reversed phase - High Performance Liquid Chromatography (RP-HPLC) and then incubated treated fractions and determined growth inhibition by the measure of absorbance. The secretions resulted in 42 fractions, among which two had activity, against Gram positive bacteria *Micrococcus luteus* and against yeast *Candida albicans*. The low concentrations in which the secretions were active are relevant from the biotechnological point of view. For the animals, the secretions possibly prevent infections including when they are attacked in these regions by predators that pick that spot to bite.

INTRODUCTION. The cuticle of arthropods has several functions such as a protective barrier against predators, preventing water loss, barrier against ingress of water, ions or environmental chemicals, maintaining body shape, structural support and physical barrier against parasites and microorganisms [Charnley & Leger 1991, Ortiz-Urquiza & Keyhani 2013]. Fungi, for example, have strategies that allow them to cross arthropod cuticles that include adhesion mechanisms and production of enzimes, and other metabolites that facilitate infection [Ortiz-Urquiza & Keyhani 2013]. If the physical barriers are crossed, an immune response mediated by compounds in the hemolymph, which contain hemocytes and plasma, come into play [Loker et al 2004].

The main sites of infection by microorganisms in arthropods are wounds, sense organs and arthrodial membrane (AM) at joints and between segments [Klowden 2013]. The AM are particularly great sites for infection since they are generally thinner due to the reduction or absence of the exocuticle [Leger RJ 1990, Klowden

2013]. In a recent work, we demonstrated that the arthrodial membrane of the harvester *Mischonyx squalidus* (Arachnida, Opiliones) shows features of secretory activity such as pores and cuticular canals, secretory cells with mitochondria, smooth endoplasmic reticulum and secretory granules (Silva et al 2023, accepted for publication). Furthermore, we demonstrated that the secretion of these secretory cells includes proteins and oils (Silva et al 2021). In view of this evidence and based on the literature, we suggested that the arthrodial membrane has a gland with a lubricating function at least in the studied leg joint of legs IV (Silva et al 2023, accepted for publication). Since arthrodial membranes are easily accessible sites for invasion by microorganisms, we can expect that these animals have ways of avoiding infections. We therefore intended to test whether the secretion released by the secretory cells below the arthrodial membrane of *M. squalidus* inhibits the growth of pathogenic microorganisms, looking at specific fractions of molecules with antimicrobial activity.

METHODS *Mischonyx squalidus* (Roewer, 1913) appears as *Mischonyx cuspidatus* or *Ilhaia cuspidata* in previous papers (see Gueratto et al. 2021). We manually collected male individuals of *M. squalidus* (August 2018 and January 2020) under SISBIO/ICMBio license number 61431-1- 2018. We found the animals under trunks at the Parque Ecológico do Tietê, São Paulo city, São Paulo State, Brazil. We fed the animals twice a week with moist dog food. We extracted the secretion from pre-chilled animal (-20 ℃ for 15 min) by puncturing the dorsal region of arthrodial membrane gland with a pyrogenic syringe. We use a total of 100 µL of secretion extracted from 35 animals. We diluted the secretion in ultrapure water (50 µL) and trifluoroacetic acid (TFA) 0,05% (50 µL). To quantify the gland secretion molecules, we quantified proteins and peptides by reading the absorbance at 280 nm and 205 nm, using 1 µL of sample in the spectrophotometer NanoDrop 2000 model (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) full spectrum (190 to 940 nm). To fractionate the secretion from the arthrodial membrane gland in *M. squalidus* (Fig. 1), we injected the secretion dilution (200 µL) in 0.05% TFA (18000 µL). We fractionated and analyzed the samples in a Reversed phase - High Performance Liquid Chromatography (RP-HPLC) (Shimadzu LC-8A). We ran the analysis in a 60 min gradient at a 1 mL/min flow rate, with a C18 analytical column (Jupiter, 4.6 mm ×

250 mm) equilibrated with 0.05% trifluoroacetic acid (TFA) at ambient temperature. The elution gradient for the sample was 0−80% of solution B (Acetonitrile acidified with 0.05% Trifluoroacetic acid) in solution A (0.05% Trifluoroacetic acid). We monitored the effluent absorbance at 225 nm, and the fractions were hand collected, concentrated under vacuum, and reconstituted in ultrapure water. To assess whether AM secretion has the ability to inhibit the growth of the microorganisms, we conducted the liquid microbial growth inhibition assay in 96-well microplates. We concentrated each fraction of secretions obtained via RP-HPLC in 100 µL of ultrapure water. **Microbial Strains** – We obtained bacterial and yeast strains from the collection of microorganisms of the Laboratory for Applied Toxinology (LETA) of the Butantan Institute (São Paulo, Brazil). We performed the bioassays with *Micrococcus luteus* A270, *Escherichia coli* SBS363 and *Candida albicans* MDM8. **Antimicrobial Assays** – We evaluaetd the antimicrobial effects by liquid growth inhibition assays as described in Hayashida and Silva Junior (2021). We cultured bacteria in poor nutrient broth (PB) (1.0 g of peptone in 100 mL of water containing 86 mM NaCl at pH 7.4; 217 mOsM), and yeast in poor potato dextrose broth (1/2 PDB: 1.2 g of potato dextrose in 100 mL of H2O at pH 5.0; 79 mOsM). We determined antimicrobial activity using a five fold microtiter broth dilution assay in 96-well sterile microplates at a final volume of 100 µL. We diluted mid-log phase culture to a final concentration of 5×10^4 CFU/mL for bacteria and 5×10^5 CFU/mL for yeast (Hayashida and Silva Junior 2021). We dissolved dried fractions in 100 µL of ultrapure water, and placed 20 µL aliquots in each well with 80 µL of the microbial dilution. We incubated the microplates for 18 h at 30 °C; we determined growth inhibition by measuring absorbance at 595 nm. As a positive control of microbial growth inhibition, we used 10 mg/mL of antibiotics (ampicillin, streptomycin and tetracycline).

RESULTS

The secretions resulted in 42 fractions via HPLC (we tested all fractions for antimicrobial activity), among which two had antimicrobial activity (Fig 2). Fraction 59 was active against Gram-positive bacteria *Micrococcus luteus* and fraction 71 was active against yeast *Candida albicans* (Table 1). None of the molecules was active against Gram-negative bacteria *Escherichia coli* (Table 1). Fraction 71 was more concentrated than fraction 59 (Table 2).

DISCUSSION

A number of papers describe antimicrobial molecules in setae, venom, secretions, hemolymph (hemocytes and plasma) and body extracts in arthropods, including arachnids (Riciluca et al 2021; Chaparro & Silva-Junior 2016; Diaz-Roa et al 2018; Segura-Ramírez & Silva-Junior 2018). In harvesters, Sayegh et al (2016) found an AMP (longipin) in the hemolymph of *Acutisoma longipes* (Opiliones, Gonyleptidae) that has antifungal activity. However, to our knowledge, ours is the first study finding antimicrobial molecules beneath the arthrodial membranes in arachnids.

Harvesters in the suborder Laniatores are known for their thick integument that protect them against predators (Souza and Willemart 2011; Dias and Willemart 2013). One of the vulnerable areas are precisely the arthrodial membranes of legs, which are used by specific predators to bite (Segovia et al 2015). In such cases, should the harvester escape the attack, it will have a wound that gives free access to bacteria and fungi (Leger 1990; Charnley & Leger 1991). Therefore, the antimicrobial molecules we found are probably of importance in this context.

The proteomic analysis of the content of the secretory cells in the AM revealed similarities with proteins, specifically with peptides with antimicrobial activity [Silva et al 2021], whose activity have now been demonstrated. Moreover, both fractions have antimicrobial activity at low concentrations, which is important from a pharmacological point of view (Nascimento et al 2016; Diniz et al 2018; Diaz-Roa et al 2018, 2019).

The function of the other proteins remains unknown, but Silva et al (2021) have found molecules putatively homologous to peptides and/or proteins with functions such as cellular metabolism, signaling and binding and defense. We also do not know the mechanisms through which the peptides act, but they usually either break the cell membrane or interact with internal components of the bacterium cell (Benfield and Henriques 2020).

In conclusion, we found an antiseptic function of the secretions in the arthrodial membrane of the leg IV of a harvester, a region particularly vulnerable to infections. Because of its low concentration and efficiency against a bacterium and a fungus, there is also potential pharmacological interest.

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FIGURE LEGENDS

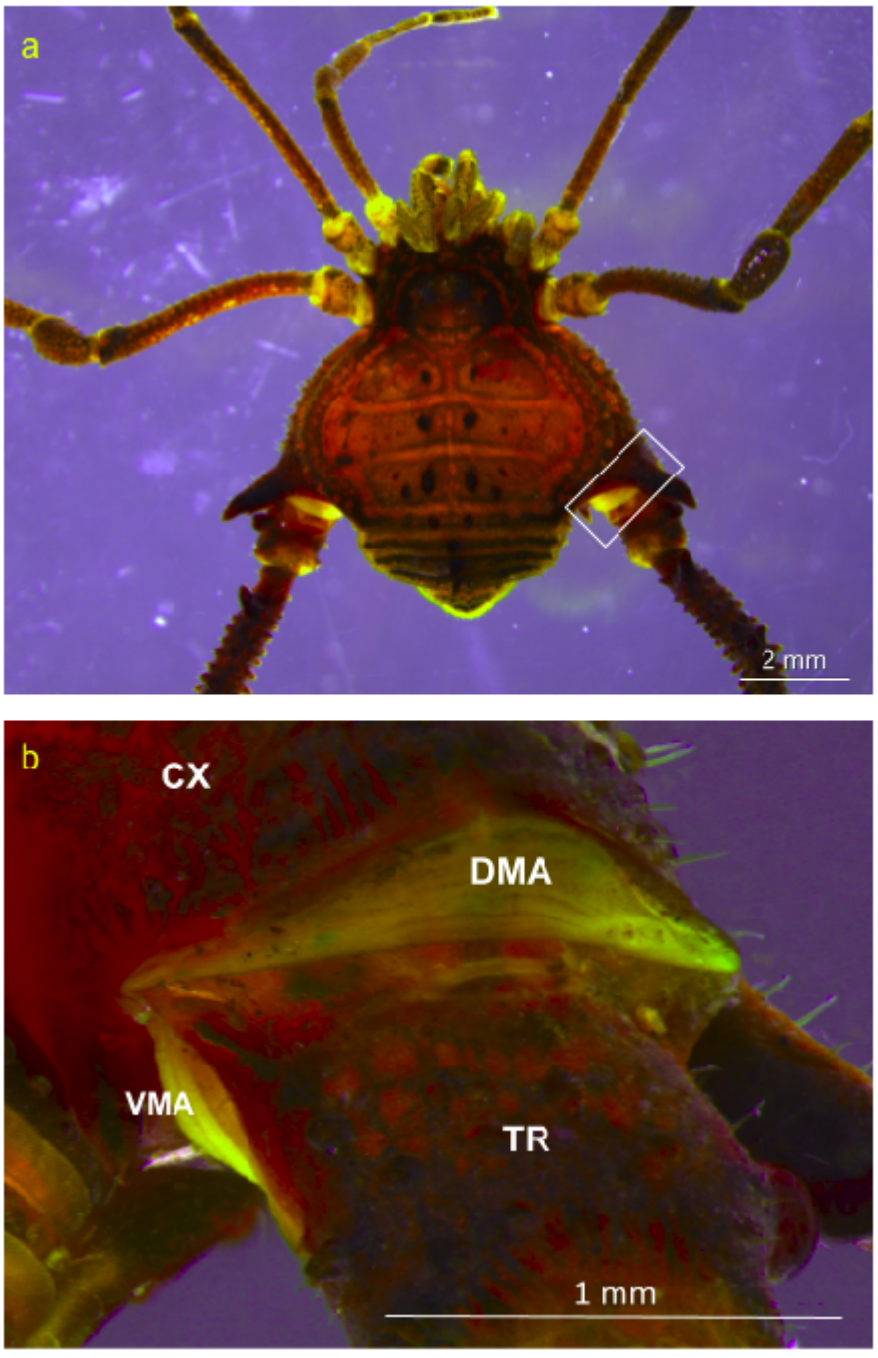

Figure 1. a) Dorsal view of a male in the harvester *Mischonyx squalidus* (Arachnida, Opiliones). The white square shows the arthrodial membrane on a leg IV. b) Detail of the arthrodial membrane in the ventral and dorsal regions. cx = coxa, tr = trochanter, dma = dorsal arthrodial membrane, vma = ventral arthrodial membrane.

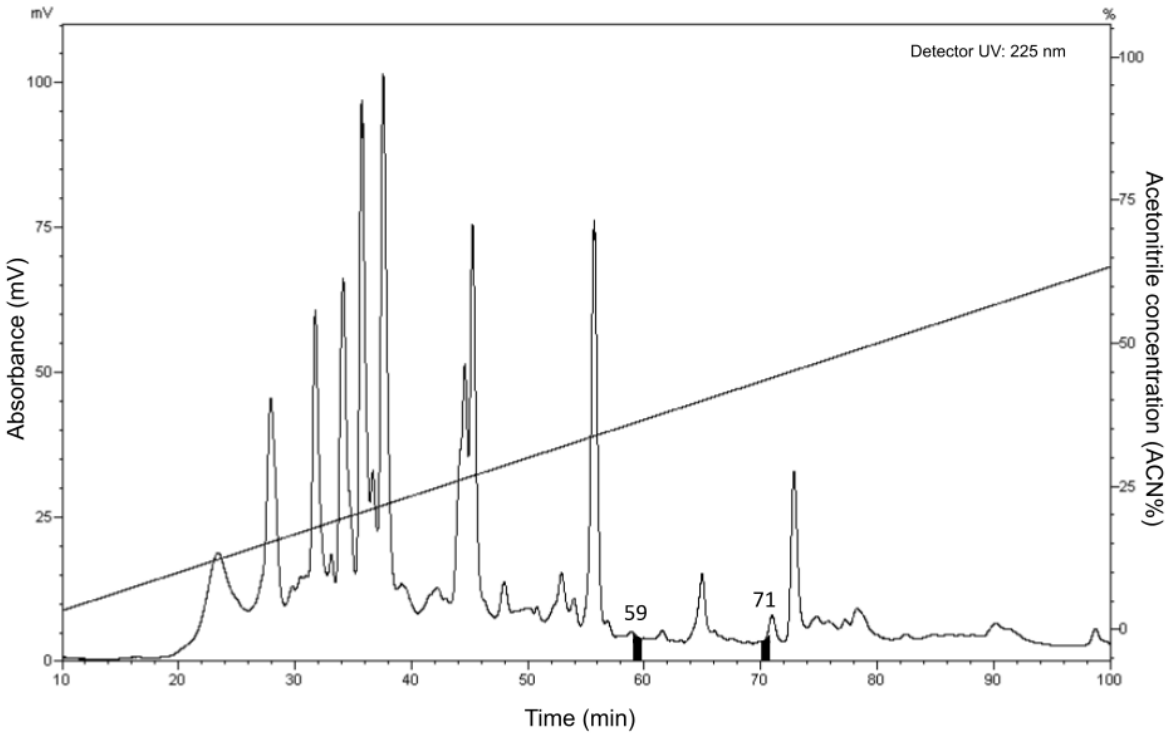

Figure 2. Chromatographic profile of the purification step of the harvester *Mischonyx squalidus* (Arachnida, Opiliones) arthrodial membrane gland secretion. High performance liquid chromatography in reversed-phase (RP-HPLC) analysis was performed in a 60 min gradient at a 1 mL/min flow rate (Jupiter C18 analitical column, 4.6 mm × 250 mm) equilibrated with 0.05% trifluoroacetic acid (TFA). The enumerated peaks correspond to the fractions with antimicrobial activity.

TABLES

Table 1. Antimicrobial activity in the secretion of an arthrodial membrane of the harvester *Mischonyx squalidus* (Arachnida, Opiliones), analyzed by high-performance liquid chromatography in reversed-phase using Shim-pack XR-ODS C18 analytical column of secretion treated with 10% DMSO, with flow 2.0 mL / min, in 60 min; 225 nm absorbance. Tests carried out against *Micrococcus luteus* A270, *Escherichia coli* SBS 363 and *Candida albicans* MDM8. (+) inhibition of antimicrobial activity and (-) non-inhibition of antimicrobial activity.

Microorganisms		Fractions	
		59	71
Gram-positive bacteria	*Micrococcus luteus* A270	+	-
Gram-negative bacteria	*Escherichia Coli* SBS 363	-	-
Yeast	*Candida albicans* MDM8	-	+

Table 2. Concentration of proteins and peptides present in fractions (59 and 71 by RP-HPLC) of the secretion from the gland of the arthrodial membrane of the harvester *Mischonyx squalidus* (Arachnida, Opiliones). The absorbance reading (280 nm and 205 nm) was conducted using a NanoDrop 2000 model spectrophotometer.

Fractions	A280nm	A205nm
59	0.003 µg/µL	0.003 µg/µL
71	0.025 µg/µL	0.026 µg/µL

CAPÍTULO 4

Cuticular hydrocarbons of *Mischonyx squalidus*, a Neotropical harvester (Arachnida, Opiliones)

Cuticular hydrocarbons of *Mischonyx squalidus*, a Neotropical harvester (Arachnida, Opiliones)

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ABSTRACT

Cuticular hydrocarbons were examined in harvester *Mischonyx squalidus*. The total content of cuticular hydrocarbons from male and female individuals was extracted with hexane and the compounds were identified through gas chromatography – mass spectrometry (GC-MS). The results showed eleven and ten cuticular hydrocarbons

for female and male individuals, respectively. The identified compounds belong to the three major classes of hydrocarbons found in insects, including n-alkanes, methyl-branched alkanes, and alkenes. Methyl-branched pentadecane and hexadecane were the hydrocarbons with the higher relative percentual found in the cuticle of *M*. *squalidus*. Among these compounds, heneicosane was exclusively found in female individuals and, here, we suggested this hydrocarbon as a compound used in the partner recognition. The other compounds found are typically used in chemical communication in other animals, which is consistent with the sensory biology of harvesters.

KEYWORDS: harvestman, harvestmen, chemical communication, contact pheromone

INTRODUCTION

Cuticular hydrocarbons (CHCs) are universal constituents of wax in the cuticle of arthropods (Howard et al. 2005), which often possess complex mixtures of hydrocarbons that belong to the three main classes of hydrocarbons, including, n-alkanes, methyl-branched alkanes, and alkenes (Blomquist and Bagnères 2010). The size of a hydrocarbon chain ranges from approximately 20 to 50 carbon atoms (Blomquist and Dillwith 1985). The primary function is protection against water loss: species that live in dry environments (sandy deserts, savannahs, etc.) contain longer hydrocarbon chains than their relatives in humid conditions, which is assumed to help preventing water loss (Blomquist and Dillwith 1985, Lockey 1988). However, CHCs play an important role in communication, such as intra and interspecific recognition (Wyatt 2014)..

CHCs play critical roles in chemical mimicry, dominance and fertility cues, task specific cues, species and gender recognition cues, primer pheromones, kairomones, nestmate recognition and sex pheromone (Blomquist et al 1998, Clément and Bagnères 1998). In this context, in many species, CHCs are responsible for recognizing males and females for mating (Blomquist and Bagnères 2010). The recognition of hydrocarbons is possible due to the difference in one or more compounds existing in the cuticles of these animals (Blomquist and Bagnères 2010). Arthropods typically touch the cuticle of another individual with chemosensory

organs present on the antenna of insects and legs and pedipalps in arachnids (Foelix and Chu-Wang 1973, Abdalla and Cruz-Landin 2001, Ozaki and Wada-Katsumata 2010) that allow them to assess the information present in CHCs. Despite the crucial importance in chemical communication, some speciose taxa have never had their CHCs studied despite clear evidence that cuticle chemicals should be very relevant in their behavior. This is the case of arachnids in the order Opiliones, popularly known as harvestmen or harvesters.

The order Opiliones is one of the largest groups within the class of arachnids with approximately 6600 described species. Most harvesters are nocturnal and can often be found under logs, caves, rocks, plants or litter, preferentially in humid places (Acosta and Machado 2007, Curtis and Machado 2007). Mate finding and the mode of recognition between sexual partners is not known, but chemicals most probably play an important role (Fernandes and Willemart 2014, Murayama and Willemart 2015, Fernandes et al 2017a). Copulation is often triggered by direct contact with some region of the female's cuticle (Fowler-Finn et al 2014) and there may be a lot of contact between chemoreceptors and the cuticle during copulatory and pre-copulatory courtship (Willemart et al 2006; 2009a; Requena and Machado 2014; Stanley et al 2016) or male-male interactions (eg Willemart et al 2009b; García-Hernandez and Machado 2018). Despite clear evidence of the importance of contact and probably chemicals in conspecific communication, there has not been a single study on the CHCs of harvesters. We therefore asked whether such chemicals would match previously described CHCs that are used in conspecific communication in other animals. Thus, our objective was to characterize the CHCs of males and females of the harvester *Mischonyx squalidus* Rower 1913 (Arachnida, Opiliones, Gonyleptidae).

MATERIAL AND METHODS

Mischonyx squalidus (Roewer, 1913) appears as *Mischonyx cuspidatus* or *Ilhaia cuspidata* in previous papers (see Gueratto et al 2021). We manually collected male and female individuals of *M. squalidus* in February 2022 under SISBIO/ICMBio license number 61431-1- 2018. We found the animals under trunks at the Parque Ecológico do Tietê, São Paulo City, São Paulo State, Brazil. We made the extractions between one and three days after collecting the animals in the field.

Cuticular Hydrocarbon Analysis

To verify if there are differences in the chemical composition of the cuticular hydrocarbons of *M. squalidus* we added 10 males and 10 females separately in 1500 µL of hexane for 5 min. We evaporated the extracts using nitrogen gas and added 20 µL into a 200-µL insert. Extracts were analyzed by gas chromatography (6850 Network GC System, Agilent) coupled with mass spectrometry (Agilent 5975C VL MSD) (GC-MS) equipped with a HP5-MS capillary column (Agilent, length 30 m, ID 250 µm, 0.25 µm film thickness). The initial column temperature was adjusted to 100°C for 5 min, and ramped at 5°C min^{-1} to a final temperature of 320°C, with a total run time of 57 min. The injection volume was 1 µL with helium as a carrier gas at 1 $mL.min^{-1}$. The injector, ion source, and quadrupole temperatures were 300ºC, 280ºC, and 180ºC, respectively. MS detection was performed with electron ionization (EI) at 70 eV, working in the full-scan acquisition mode ranging between 50-800 *m/z* at 2.66 scan s^{-1}. Compounds were identified by comparison of mass fragmentation using NIST digital library spectra 2.0 (2008) and the fragmentation pattern. n-Alkanes were characterized based on comparison of retention times with components of a homologous series in the range C_{19}–C_{40} (Carvalho et al 2021). Hydrocarbon patterns (C_{19}–C_{40}) were also analyzed via GC-MS.

RESULTS

According to the GC-MS analysis was observed thirteen peaks in the chromatogram of hexane extract of which eleven compounds were identified in females and ten in males of the harvester *Mischonyx squalidus* (Table 1). Of the total six compounds belonging to the n-alkanes, four belong to the methyl-branched alkanes and one to the class of alkenes. Methyl-branched pentadecane and hexadecane were the most abundant cuticular hydrocarbons found in both groups of individuals (Table 1). Females showed only one cuticular hydrocarbon (heneicosane) that was absent in males (Table 1).

DISCUSSION

Our study provided the first description of cuticular hydrocarbons (CHCs) in the order Opiliones. We identified thirteen cuticular hydrocarbons extracted from harvesters of the species *M. squalidus*. We found CHCs compounds belonging to the

three major classes of insect hydrocarbons which include, n-alkanes, methyl-branched alkanes, and alkenes. Principal component analysis of thirteen CHCs peaks from *M. squalidus* revealed a weak difference and proportion across male and female CHCs.

Methyl pentadecane and hexadecane compounds accounted for more than 50% of the total chemical present.

Most of the CHCs described in our study were also found in insects and arachnids. Nonadecane, heneicosane, tricosane and pentacosane, for example, have been found in two species of desert scorpions (Trabalon and Bagnères 2010). Pentacosane and two other compounds of the methyl-branched alkanes class were found only in females or in low concentrations in male beetles. All three compounds were needed to elicit a complete sequence of mating behavior in males (Ginzel et al 2003). Pentacosane is also known as a fertility signal and is probably a pheromone used to signal reproduction in bees (Pizzi and Rehan 2021). Single alkenes and also long chain length analogs are molecules used as contact pheromones in beetles (Ginzel 2010).

Differences in CHC profiles across males and females are to be expected, since arachnids are expected to differentiate preys, enemies, males and females based on chemosensory or tactile signals to communicate (Bagnères et al 2001, Polis 2001, Trabalon and Bagnères 2010). Although the cuticle of arthropods contains a complex mixture of hydrocarbons, in many cases only a few compounds are part of the contact pheromones of a given species (Blomquist and Bagnères 2010). Heneicosane was the only CHC that differed between males and females studied here. Heneicosane is an alkane that has 21 carbons and a straight-chain structure. This compound has been found in plants and insects. It has a role as a plant metabolite, an oil component and a pheromone (Fernandes et al 2017b). In social insects the presence of this compound has been identified as a signal for both age and reproductive status. Pre-reproductive bees and virgin females overexpress three compounds including, farnesol, nonadecane and heneicosane (Mas and Jallon 2005, Pizzi and Rehan 2021). The heneicosane compound has also been suggested as a royal pheromone in pre-reproductive neotenic termites, queens and kings (Funaro et al 2018, Eyer et al 2021). It is possible that the heneicosane compound

serves as a female recognition pheromone in *M. squalidus*, as this species is nocturnal and likely requires chemicals for mate recognition.

Our results clearly suggest that the CHCs found to play a role in chemical communication.

Pentadecane, hexadecane and tetradecane are commonly used in chemical communication, including contact recognition, as a pheromone (and sometimes as an allomone) in insects and spiders (Trabalon and Bagnères 2010). 1-Hexadecene has been described in pheromones in insects and primates, nonadecane in insects and Squamata (“Reptilia"), and heneicosane, tricosane and tetracosane in insects, spiders and Squamata. Pentacosane is part of a pheromone in insects and spiders (Scordato et al 2007, Trabalon and Bagnères 2010, Trabalon 2011, Khannoon 2016). Therefore, adding to the paragraphs above, these results also suggest that the CHCs found play a role in chemical communication.

From a behavioral perspective, harvesters have been shown to be clearly dependent on chemicals to find resources, be it by contact chemoreception (Willemart & Chelini, 2007; Willemart et al., 2009a) or olfaction (Costa & Willemart, 2013; Santos et al 2013; Costa et al., 2016). Although there is evidence of olfaction being used to locate conspecifics (Dias et al 2020), contact has been shown to be necessary for mate recognition (Fernandes et al 2017a). From a morphological perspective, there is evidence of both contact chemoreception and olfaction (Willemart et al., 2009a; Gainett et al 2017, 2018. The missing piece was precisely the chemical one. With this study, we have shown that CHCs found in the harvester *M. squalidus* have been known to play a role in chemical communication in several taxa. With so many studies on interactions between conspecifics in harvesters (eg Dias and Willemart 2016; Harvey et al 2017; Escalante et al 2022; Palaoro et al 2022) we hope to have opened a door toward studies on how chemicals matter in such interactions.

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Table 1. Cuticular hydrocarbons identified by GC-MS in males and females of the harvester *Mischonyx squalidus* (Arachnida, Opiliones). Extracts were obtained with hexane and analyzed by gas chromatography coupled with mass spectrometry. Hydrocarbon patterns (C19–C40) were also injected.

Compound	Retention Time (min).	Male (% área)	Female (% área)
Methyl-branched tetradecane	10.007	5.4 – 5.8	5.2 – 5.7
Methyl-branched tetradecane	10.164	4.1 – 5.0	4.0 – 4.4
Tetradecane	10.495	4.9 – 5.8	4.7 – 5.4
Methyl-branched pentadecane	11.441	48.1 – 51.8	46.7 – 51.6
Methyl-branched hexadecane	16.090	1.0 – 1.1	1.0 – 1.3
Hexadec-3-ene	16.683	2.2 – 2.3	2.2 – 2.3
Methyl-branched hexadecane	16.945	16.8 – 17.5	16.2 – 17.7
Nonadecane	23.399	2.3 – 2.4	2.3 – 3.4
Heneicosane	27.367	-	1.2 – 1.3
Tricosane	30.531	3.6 – 4.1	3.2 – 4.1
Tetracosane	32.258	2.9	2.4 – 2.8
Pentacosane	34.050	3.2 – 3.8	3.1 – 3.9
Methyl-branched pentacosane	34.251	2.3 – 2.5	2.2 – 2.7

CONCLUSÃO GERAL

Esperamos ter contribuído para o conhecimento acerca dos opiliões e do funcionamento de artrópodes em geral, abrindo portas para uma gama de estudos futuros inclusive na área biotecnológica por conta da ação antifúngica e antibacteriana de secreções. A comunicação química também é uma área de importância crescente em opiliões dada a quantidade de estudos comportamentais que relatam indivíduos utilizando o contato para obter e transmitir informações para co-específicos. Quanto à minha formação, almejava, no início do doutorado, não apenas obter o título, treinar habilidades específicas de pesquisadores e contribuir com o conhecimento nos assuntos estudados, mas em particular aprender técnicas de laboratório que desconhecesse. Tendo atuado em todas as etapas do desenvolvimento de uma pesquisa, com os 4 capítulos (e anexo 1) bem encaminhados e tendo utilizado variadas técnicas, penso que os objetivos foram felizmente cumpridos.

ANEXO 1

Anexo 1 - Estudo realizado e publicado durante o período do doutorado no ICB. Norton Felipe dos Santos Silva, Guilherme Ferreira Pagoti, Rodrigo Hirata Willemart, 2020 Water locomotion and survival under water in a riparian harvestman (Opiliones, Arachnida), Behavioural Processes, Volume 179, 104220, ISSN 0376-6357, https://doi.org/10.1016/j.beproc.2020.104220.

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Water locomotion and survival under water in a riparian harvestman (Opiliones, Arachnida)

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ABSTRACT

Animals that live by rivers may benefit from being able to cross them, but behavioral adaptations are needed. Additionally, being able to remain submerged is also important if the animal moves under water. Here we asked whether the harvestman *Heteromitobates discolor* (Opiliones), that lives by rivers, (a) can propel itself across the water surface, (b) moves onto the water if disturbed and (c) can survive for long periods when submerged. *Heteromitobates discolor* exhibited two gaits on water, whereas a strictly terrestrial species was not able to propel itself. When experimentally submitted to simulated predator attack on a rock on the river, *H. discolor* walked onto the water, while a strictly terrestrial species did not. Finally, it was able to survive for 6 h under water, presumably due to the conspicuous air film that formed around its body, which was also observed in a strictly terrestrial species. Altogether, these observations suggest that the aquatic environment is not a barrier for regular activity and can be used as an extension of the terrestrial environment for *H. discolor*.